CANCER RESEARCH

VOLUME 11 SEPTEMBER 1951 NUMBER 9

Differences in Immunologic Reactions of Red Blood Cells of a Tumor-susceptible (C3H) and a Tumor-resistant (C57) Mouse Strain*

LUCIE ADELSBERGER

(Laboratory Division of Montefiore Hospital, New York, N.Y.)

The development of the immune hemagglutination technic and recent observations of immunological differences between strains of mice susceptible and resistant to neoplasms suggested the application of this technic to the study of mammary cancer in mice, with the use of antiserum from a heterologous species. Thus, Gorer (8) observed that Strong's A strain of mice, highly cancer-susceptible animals, possesses two iso-antigens that are shared by the erythrocytes and a number of fixed tissues, including all neoplastic tissues studied thus far. He did not find iso-antigens in the tumor-resistant Little C57 black strain of mice. Members of this strain immunized against murine neoplasms showed an excellent antibody response against the tumor and against the red blood cells from A mice. Kaliss and Jay (11) used the same tumor as Gorer but separated the tumor taken from A mice from the A red blood cells by intermediary grafting into other strains. They were thus able to demonstrate that agglutination of the A red blood cells did not occur with the sera from the tumor-immunized C57 black, except when the C57 mice got their tumors from Street mice.

Davidsohn and Stern (4, 5) found that 90 per cent of the C57 black mice possessed natural agglutinins against sheep erythrocytes, whereas the incidence and titer of these agglutinins in the other strains were much lower. The low incidence and titer of these agglutinins of three tumor-susceptible stocks (C3H, A, and D2), as shown by Davidsohn, Stern, and Bittner (6), was not influenced by the presence or absence of the milk agent. In mice of the I stock, a resistant strain, results comparable to those of cancerous strains (6) were noted.

The present paper is a report of observations on the agglutination of erythrocytes from a mouse strain susceptible and a strain resistant to mammary cancer by serum from rabbits previously injected with cell-containing filtrates from homologous tumor tissue.

MATERIALS AND METHODS

Mouse strains and tumors.—Two inbred strains of mice were used, the high tumor-susceptible C3H strain and the tumor-resistant C57 black strain. Most of the animals came from our own breeding colony, originally obtained from Dr. L. S. Strong at Yale, and had been inbred for nearly 4 years. The mammary cancer in C3H mice was the tumor employed in this study; only spontaneous neoplasms were used except in some *in vitro* tests.

Preparation of antigen.—The tumor was used either immediately after the death of the mouse or after storage at −20° C. for varying lengths of time. It was weighed, ground in a mortar with sterile sea sand, and 0.85 per cent saline was added to make a final dilution of 10 per cent. The suspension was filtered through a silk mesh with 200 openings per square inch. This was substituted for the disc in an E. K. Seitz filter. The entire procedure, which lasted from 30 to 55 minutes, was performed under sterile conditions on ice. The tumor silk filtrate (T.S.F.) was a homogeneous, slightly turbid, sometimes somewhat hemorrhagic fluid

* Aided by a grant from the Damon Runyon Memorial Fund.

Received for publication February 16, 1951.

which contained tumor cells on microscopic examination.

Immunization of rabbits.—An immunization method similar to that of Andervont (1) was employed. Seventeen male rabbits were given one or two intravenous injections of 1.0 cc. T.S.F. followed by two to three weekly intraperitoneal injections of 2–3 cc. T.S.F., according to the amount available. Three rabbits were injected in the same manner with a Seitz filtrate, which had been procured from the T.S.F. and was given either alone or mixed with equal amounts of rabbit serum diluted 1:10 (two animals). Another rabbit was injected intracutaneously with liquefied aquaphor, a cholesterolized absorption base, and T.S.F. by a method similar to that of Freund (7). This animal received ten injections, twice weekly, of 0.1 cc. aquaphor and 0.1 cc. T.S.F. The progress of immunization was tested by using blood from the ear; serum in quantity was prepared from heart blood taken at various periods, optimally 2–4 weeks, after the last injection.

Sera stored at 4° C. were tested within 3–4 days after preparation, or they were maintained for longer intervals at −20° C. The complement of the sera was inactivated by heating at 56° C. for 30 minutes.

Hemagglutination.—The red blood cells were generally pooled from several mice. The C3H red blood cells were from female mice, breeders, at least 6 months old and, in most instances, older. The C57 blood cells were secured from old C57 males. The mice were bled from the tail, and the blood was collected in 3.5 per cent sodium citrate or in Alsever's solution. The erythrocytes, separated by centrifugation for 4 minutes at a speed of 3,250 r.p.m., were washed 2–3 times with cold, sterile saline and stored at 4° C. In the first experiments they were used within 24 hours, in later ones always within 4–8 hours. They were diluted to a 1 or 1.5 per cent saline suspension which were equally used for these tests.

In the test a series of ten tubes containing 0.2 cc. of twofold serum dilutions (1:2 to 1:1024) in saline and 0.2 cc. of red blood cell suspension was prepared. The tubes were incubated at 4° C. or kept for 30, 60, or 90 minutes at 37° C. and then in the refrigerator. Some tests were carried out at room temperature. Readings were taken at $1\frac{1}{2}$–2 hours and again at 18–24 hours, sometimes more frequently. The degree of agglutination was determined by direct inspection of the bases of the tubes and by evaluation of the clumps. The formation of a compact disc on the bottom of the tube or one single, heavy clump was recorded as 4 plus, several large coarse aggregates as 3 plus, a large number of smaller ones as 2 plus. Fine particle agglutination which was easily recognized with the naked eye was evaluated as 1 plus. The hemagglutination titer was taken as the highest dilution of serum with which a 1 plus agglutination was read.

Parallel tests with the same serum were conducted with red blood cells from nontumorous C3H and C57 and tumor-bearing C3H mice. Thirty-one sera from normal rabbits were used as controls.

RESULTS

Hemagglutination of antitumor rabbit sera with red blood cells from nontumorous mice.—Eighteen antitumor rabbit sera were tested for agglutination of mouse erythrocytes in 104 tests. The resulting agglutination showed characteristic features observed with all antitumor sera tested, including the serum prepared by intracutaneous injections. The hemagglutination titer of C3H red blood cells averaged 1:128, or twice that of C57 red blood cells. This titer was seen at the various temperatures of incubation. (At $1\frac{1}{2}$–2 hours it was occasionally twofold lower in tests performed in the cold than in tests performed at 37° C.) Thus, there was no significant difference observed in the titers of the C3H and C57 erythrocyte agglutination in these tests. However, there was observed a difference in the rate and pattern of the parallel tests, as is demonstrated in Chart 1. Agglutination of the C57 erythrocytes occurred as a rather constant and regular phenomenon. Records taken at 2-hour and at 18–24-hour readings were approximately the same and demonstrated an agglutination which was gradually decreasing with increasing dilutions of antitumor serum. On the other hand, a mid-zone of weak agglutination was characteristically found with C3H red blood cells in the 1:8 and 1:16 dilutions of the antitumor sera. Occasionally the phenomenon was observed at lower or higher dilutions. Furthermore, the mid-zonal weak agglutination was in general transitory in such tests and was noted either early or late during the period of observation. It consequently made the C3H erythrocyte agglutination a rather unstable one. It failed to appear under the following conditions: (*a*) heating of the serum to 60°–62° C. for 20 minutes, (*b*) storage of the red blood cells overnight, and (*c*) storage of the serum for a period longer than 4 days at 4° C. The weak C3H erythrocyte agglutination was seen regularly in eleven of the antitumor sera; with the other seven, it was observed less frequently. At present, it is questionable whether this is due to the red blood cells or the serum or both. Although the phenomenon was evi-

dent at various temperatures of incubation, it was most clearly demonstrable in some experiments with cold incubation. It was best demonstrated with sera procured 3–4 weeks after the last immunizing injection. With sera obtained at longer time intervals, the mid-zonal phenomenon was lost.

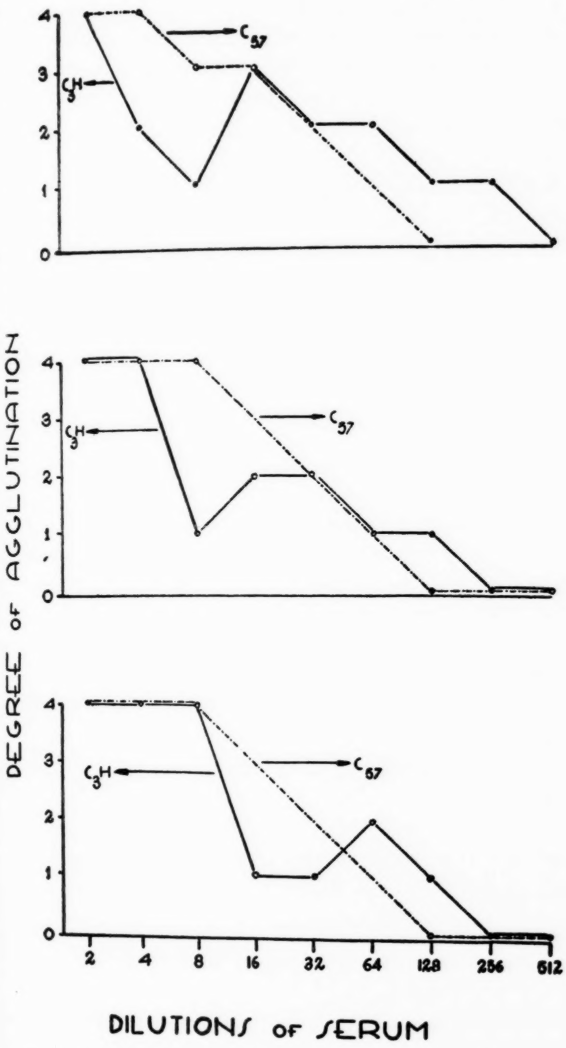


CHART 1.—Hemagglutination of antitumor rabbit serum with red blood cells from C3H and C57 mice showing mid-zonal weak C3H agglutination (results of three typical experiments).

The results obtained from the sera of three rabbits which had been injected with tumor Seitz filtrate were doubtful. One of these sera revealed the weak C3H agglutination for a period of 3 months after the last immunizing injection.

Normal rabbit sera agglutinated, in general, the red blood cells from both strains of mice to a titer of 1:8 or 1:16. Serum dilutions of 1:4 or 1:8 frequently produced a relatively weaker C3H agglutination, but the reverse was also seen.

Agglutination of antitumor rabbit sera with the red blood cells from tumor-bearing mice.—Results different from those described above were observed in 31 out of 35 tests in which the red blood cells were obtained from C3H mice with spontaneous tumors. In these tests the mid-zonal phenomenon was not present (Chart 2). The agglutination decreased gradually with increasing dilutions of antitumor serum. It was also found to be more stable than the agglutination of erythrocytes from nontumorous C3H mice. It developed slowly and, in several instances, after more than 2 hours; once it had developed, it persisted throughout the observation period. It resembled more closely the C57 rather than the C3H red blood cell agglutination.

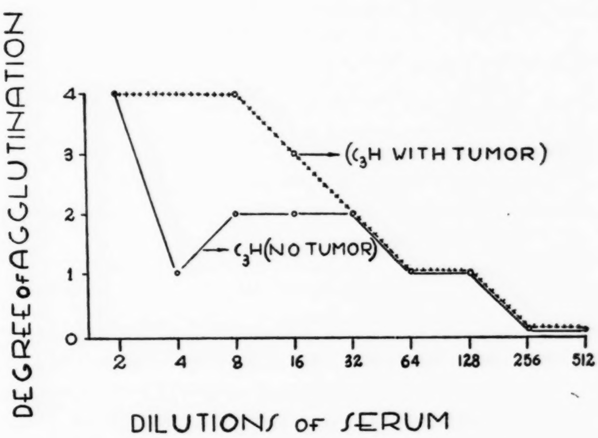


CHART 2.—Hemagglutination of antitumor rabbit serum with red blood cells from C3H mice with and without spontaneous breast tumor.

In 21 tests with red blood cells from C3H mice with transplanted tumors the agglutination, in some instances, was similar to that obtained with red blood cells from nontumorous C3H mice and in others resembled that with red blood cells from mice with the spontaneous tumors.

Hemagglutination of mixtures of antitumor rabbit serum and tumor suspension with red blood cells from nontumorous mice.—The effect of preliminary incubation of antitumor rabbit serum with tumor suspension upon the agglutination of erythrocytes from nontumorous C3H and C57 mice was then tested. A freshly prepared 10 per cent tumor suspension was employed. In 9 earlier experiments the tumor silk filtrate (T.S.F.) was used, in 27 later tests the supernatant of a tumor suspension centrifuged for 5 minutes at 3,250 r.p.m. Either one-tenth cc. of this suspension or 0.1 cc. of the supernatant was added to 0.1 cc. of twofold serial dilutions of antitumor rabbit serum. The mixtures were incubated for 15–30 minutes at 37° C. or at

4° C., and 0.2 cc. of red blood cell suspension was added. Agglutination tests were performed as previously described. Since the tumor suspension occasionally formed a sediment, final readings of hemagglutination were confirmed microscopically. Control tests with the antitumor rabbit serum (or with the normal rabbit serum) alone and with the tumor suspension or supernatant were performed in each experiment.

In 36 such experiments hemagglutination generally occurred only in low dilutions of serum, in comparison to that observed with the antitumor rabbit serum alone. Furthermore, a difference between the reactions with C3H and C57 red blood cells with the serum-tumor mixtures was observed in low serum dilutions. Dilutions of serum of 1:4

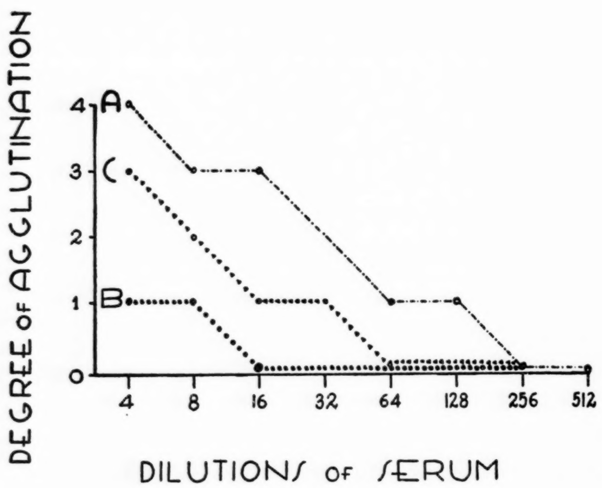


CHART 3.—Hemagglutination of C57 red blood cells with mixture of antitumor rabbit serum and tumor suspension.

A denotes C57 red blood cells with antitumor rabbit serum.

B denotes C57 red blood cells with mixture of antitumor rabbit serum and tumor suspension (2-hour reading).

C denotes C57 red blood cells with mixture of antitumor rabbit serum and tumor suspension (24-hour reading).

to 1:16 with C57 red blood cells produced a weak agglutination, or none at all, at 2 hours. In 11 tests this weak agglutination persisted throughout the entire reading period; in 25 tests agglutination increased and reached its peak at 20 hours or later (Chart 3).

In contrast, the agglutination pattern of the C3H red blood cells varied widely, in some instances resembling the gradually increasing agglutination of C57 red blood cells, whereas in others initial pronounced agglutination was present which decreased toward the 20-hour reading period.

The findings of the hemagglutination tests with the antitumor rabbit serum alone corresponded to those described above. Freshly prepared, nonmixed tumor suspensions are known to exert an agglutinating or hemolyzing effect on mouse erythrocytes (10, 13). In our experiments the tumor suspensions or supernatants produced the hemolyzing effect frequently, the agglutinating effect only occasionally. This agglutination appeared within a few hours after the red blood cells had been added and disappeared on the next day.

If normal rabbit serum instead of antitumor rabbit serum was used for the mixtures (fifteen tests), the results corresponded to those given above as to: (*a*) the gradually increasing C57 red blood cell agglutination and (*b*) the variability of the C3H agglutination.

It will be observed that the pattern of agglutination obtained with C57 red blood cells and serum-tumor mixtures resembled that previously described with C3H red blood cells in serum alone.

DISCUSSION

These experiments with the agglutination of mouse red blood cells and antitumor rabbit serum prepared against mammary cancer of mice reveal an immunological difference between the erythrocytes of a tumor-susceptible (C3H) mouse strain and a tumor-resistant (C57) strain. This difference was manifest not in the agglutination titer of the antiserum, which was essentially the same for the C3H and C57 red blood cells, but in the pattern of agglutination. Contrary to what might be expected if an antigen hereditary in the mouse were the determining factor, the agglutination of red blood cells from the C3H strain (from which the tumor for immunization was obtained) in most instances exhibited a mid-zonal phenomenon of weak agglutination. Explanation of this phenomenon at present is difficult on the basis of existing evidence. However, the rabbit immune serum undoubtedly contained antibodies against normal mouse tissues as well as against the mammary cancer as such, and the agglutination observed at 37° C. and also at 4° C. may thus be the resultant of a number of antigen-antibody reactions. Theoretically, if one observed the reaction of a normal anti-erythrocyte antibody and an agglutinin-inhibiting antibody occurring concomitantly, the irregular inhibition of agglutination seen in these experiments with C3H red blood cells might occur. One would then assume that the agglutinin-inhibiting reaction did not occur when C57 red blood cells were substituted in the hemagglutination test. Gorer (9), who has observed a pro-zone of complete inhibition of the agglutination of A strain mouse red blood cells with sera from hyperim-

munized C57 mice with tumor growth and an enhancement of this agglutination by normal serum or plasma, attributes the phenomenon to blocking antibodies. Whether this holds true for the mid-zonal weak C3H erythrocyte agglutination needs further investigation. In view of the previous demonstration of viral etiology of mouse mammary cancer (2, 3, 12, 14), another possible explanation of the hemagglutination pattern relates to the presence of the virus in the red blood cells. This view is, however, at present unsupported by experimental evidence.

Furthermore, and perhaps more importantly, the loss of the weak C3H erythrocyte agglutination in experiments with red blood cells from tumor-bearing animals of the same strain indicates a relationship between this behavior of the erythrocytes and tumor growth.

The results of hemagglutination tests with mouse red blood cells and mixtures of antitumor rabbit serum and tumor suspension were remarkable as regards the initial weakness of the C57 agglutination. The latter was found to be in contrast to the initial pronounced agglutination of the antitumor rabbit serum alone. It was also evident in the rare instances where the tumor suspension alone exerted a hemagglutinating effect which appeared within a few hours. The significance of the behavior of tumor suspension-serum mixtures remains to be explained. Apparently, it is not due to a mere absorption of species-specific mouse agglutinins, because in this case the C3H red blood cells were equally or more affected.

SUMMARY

1. Hemagglutination tests with red blood cells of normal mice and antitumor rabbit sera revealed a difference between C3H and C57 erythrocytes as to rate and degree of agglutination. A more or less transitory mid-zonal weak agglutination of C3H red blood cells was observed with serum dilutions in the range of 1:4 to 1:32.

2. Red blood cells of C3H mice with spontaneous breast tumor reacted differently than red blood cells of nontumorous C3H mice. They did not show the mid-zonal weak agglutination.

3. Mixtures of antitumor rabbit serum and tumor suspension agglutinated C3H and C57 red blood cells in a different manner. The C57 agglutination was weak initially and either persisted or gradually increased. The C3H agglutination varied widely.

4. Fresh materials such as tumor, erythrocytes, and possibly serum, as well as time and temperature, are important factors for the proper application of this hemagglutination test with antitumor sera.

ACKNOWLEDGMENT

Acknowledgment is made of the advice and encouragement of Dr. H. M. Zimmerman.

REFERENCES

1. ANDERVONT, H. B. The Milk Influence in the Genesis of Mammary Cancer. A Symposium on Mammary Tumors in Mice. A.A.A.S., **22**:123–39, 1945.
2. BITTNER, J. J. The Milk Influence of Breast Tumors in Mice. Science, **95**:462–63, 1942.
3. ———. The Mammary Tumor Milk Agent. Ann. New York Acad. Sc., **46**:69–73, 1947.
4. DAVIDSOHN, I., and STERN, K. Hemagglutinins in the Serum of Mice of Low and High Mammary Tumor Strains. Proc. Soc. Exper. Biol. & Med., **70**:142–46, 1949.
5. ———. Natural and Immune Antibodies in Mice of Low and High Tumor Strains. Cancer Research, **9**:426–35, 1949.
6. DAVIDSOHN, I.; STERN, K.; and BITTNER, J. J. Milk Agent and Natural Antisheep Agglutinins in Mice of Inbred Strains. Proc. Soc. Exper. Biol. & Med., **74**:356–57, 1950.
7. FREUND, J., and McDERMOTT, K. Sensitization to Horse Serum by Means of Adjuvants. Proc. Soc. Exper. Biol. & Med., **49**:548–53, 1942.
8. GORER, P. A. The Genetic and Antigenic Basis of Tumour Transplantation. J. Path. & Bact., **44**:691–97, 1937.
9. ———. Antibody Response to Tumor Inoculation in Mice with Special Reference to Partial Antibodies. Cancer Research, **7**:634–41, 1947.
10. GROSS, L. Destructive Action of Mouse and Rat Tumor Extracts on Red Blood Cells *in vitro*. J. Immunol., **59**: 173–88, 1948.
11. KALISS, N., and JAY, G. E. Do a Transplantable Tumor and the Red Blood Cells of an Inbred Strain of Mice Have an Agglutinogen in Common? Cancer Research, **10**:227–28, 1950.
12. PORTER, K. R., and THOMPSON, H. P. A Particulate Body Associated with Epithelial Cells Cultured from Mammary Carcinomas of Mice of a Milk Factor Strain. J. Exper. Med., **88**:15–24, 1948.
13. SALAMAN, M. H. Hemagglutination by Extracts of Tumors and of Some Normal Tissues. Brit. J. Cancer, **2**:253–66, 1948.
14. SHIMKIN, M. B., and ANDERVONT, H. B. Properties and Nature of the Milk Agent in the Genesis of Mammary Tumors in Mice. Research Conference on Cancer. A.A.A.S., **22A**:97–104, 1945.

Differences in the Hemolytic Behavior of Red Blood Cells of a Tumor-susceptible (C3H) and a Tumor-resistant (C57) Mouse Strain*

LUCIE ADELSBERGER

(*Laboratory Division of Montefiore Hospital, New York, N.Y.*)

It was shown previously (1) that red blood cells of a highly tumor-susceptible and of a tumor-resistant mouse strain react differently in agglutination tests with antitumor rabbit sera. During the course of these experiments it was also observed that the erythrocytes of these two mouse strains showed a different hemolytic behavior. Therefore, a study of the hemolytic property of mouse red blood cells was initiated, and its relation to the mammary cancer of C3H mice was investigated. Experimental data and results of these studies are given in the present report.

MATERIALS AND METHODS

Mouse strains and tumor employed.—The tumor-susceptible C3H mouse, with and without tumor, and the tumor-resistant C57 black mouse were employed in the same manner as in the hemagglutination tests previously described, except that old males and females of both strains were used. In addition, 4–6-month-old hybrids, derived from C3H fathers and C57 mothers and nursed by the latter, and 4–6-month-old C57 mice foster-nursed by C3H mothers were used.

The tumor employed was the mouse mammary cancer. These were spontaneous neoplasms and transplanted tumors which had been carried for four to twenty passages.

The antitumor rabbit sera were identical with those used in the previous hemagglutination tests. The complement of the sera was inactivated at 56° C. for 30 minutes.

Hemolysis tests.—The mouse erythrocytes were secured as described previously and used within 2–6 hours after withdrawal from the animal. The red blood cells were kept on ice throughout the entire procedure, except for the period of centrifugation. A 2 per cent suspension was prepared, and 0.2 cc. of this suspension was added to 0.2 cc. of saline or to 0.2 cc. of rabbit serum or tumor suspension. The tubes were placed in the waterbath at 37° C. for 60 minutes and afterwards kept at 4° C. Readings were taken after 2–4 hours, after 18–24 hours, and then daily up to the fourth day. The results were recorded according to the degree of hemolysis, as follows: complete hemolysis was considered as 4 plus, advanced hemolysis with some intact red blood cells as 3 plus, moderate hemolysis with a circumscribed disc of red blood cells on the bottom of the tube as 2 plus, a reddish color of the supernatant as 1 plus, and a pink color as plus-minus (±).

RESULTS

HEMOLYSIS IN SALINE

Various mouse erythrocytes were tested for hemolysis in 0.85 per cent saline. The results revealed a fundamental difference in the behavior of the C3H and C57 red blood cells. In 69 experiments the C57 red cells showed no hemolysis except in one instance. The C3H red blood cells obtained from nontumor-bearing mice, however, developed a 1 or 2 plus hemolysis after 40–45 hours in about half of the tests (38 of 74 tests). Hemolysis increased to 3 and 4 plus on the third or fourth day in 16 of the 38 tests. Twenty-four tests showed no hemolysis after 40–45 hours but 3 or 4 plus hemolysis on the third or fourth day. Hemolysis failed to occur in only 12 instances. In 15 tests with red blood cells from hybrids and from foster-nursed C57 mice there was a maximal 1 or 2 plus hemolysis which occurred in a few instances. Erythrocytes obtained from C57 mice at the age of 4–6 months did not display hemolysis.

The erythrocytes from C3H mice with spontaneous breast tumor definitely showed less hemolysis than the erythrocytes from normal C3H mice. Twenty-four out of 64 samples did not show hemolysis at all, and 9 samples displayed a

* Aided by a grant from the Damon Runyon Memorial Fund.

Received for publication February 16, 1951.

weak degree of hemolysis (±). The remaining 31 samples had variable degrees of hemolysis, from 1 to 4 plus. Incidentally, a C57 female from the breeding colony developed a spontaneous breast tumor. The red blood cells from this animal showed weak hemolysis.

The erythrocytes from C3H mice with transplanted breast tumors reacted differently than those from mice bearing spontaneous tumors. Moderate or advanced hemolysis was the rule; weak or no hemolysis was rarely observed. Thus, on the whole, the red blood cells from mice with transplanted breast tumors reacted in the same manner as erythrocytes from nontumor-bearing C3H mice.

In a few instances, red blood cells taken from pregnant C3H mice without spontaneous or with transplanted breast tumors showed no hemolysis. When retested during the lactation period (when the tumors had increased in size), there were variable degrees of hemolysis.

HEMOLYSIS WITH ANTITUMOR AND NORMAL RABBIT SERA

Mouse erythrocytes were tested for hemolysis with twofold serial dilutions of antitumor rabbit serum, utilizing normal rabbit serum as a control. The tests were set up in the same manner as for the hemagglutination experiments previously mentioned, with the only difference that a 2 per cent red blood cell suspension was employed in the present experiments. The lowest serum dilution in which a plus-minus or 1 plus hemolysis occurred was taken as an arbitrary titer of hemolysis.

The results are presented in Chart 1. In 25 tests, C57 red blood cells did not show hemolysis in any of the serum dilutions except in the one instance where they also showed hemolysis in saline. By contrast, the red blood cells of non-

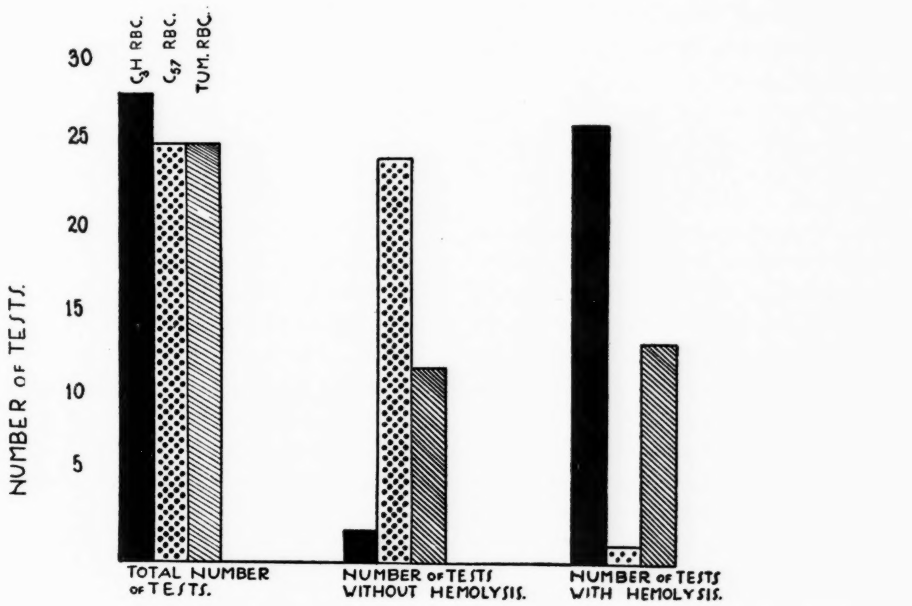


CHART 1.—Hemolysis of mouse red blood cells with antitumor rabbit sera. C3H RBC denotes red blood cells from nontumorous C3H mice; C57 RBC denotes red blood cells from nontumorous C57 mice; Tum. RBC denotes red blood cells from C3H mice with spontaneous breast tumor.

tumorous C3H mice revealed marked hemolysis in 26 out of 28 experiments with higher serum dilutions. The hemolysis started after 45–60 hours, frequently in dilutions of 1:64 to 1:256, and increased gradually involving all higher dilutions. It failed to occur with serum dilutions below 1:16.

Hemolysis of red blood cells from hybrids and from foster-nursed C57 mice occurred irregularly and was less marked than that of the erythrocytes from normal C3H mice. It developed only in some but not all the higher serum dilutions. In a few tests where red blood cells from pregnant C3H mice were used, hemolysis did not occur.

The red blood cells from C3H mice with spontaneous tumors again reacted differently than those derived from nontumorous individuals of the same strain. In 12 out of 25 tests they did not show hemolysis in any serum dilution after 45–60 hours. In the remaining 13 tests they displayed limited hemolysis in serum dilutions of 1:32 to 1:1024. However, an increased hemolysis occurred occasionally after 60–80 hours.

The red blood cells from the one C57 mouse with a spontaneous breast tumor showed weak to moderate hemolysis with antitumor rabbit serum in dilutions of 1:32 and 1:128 to 1:1024.

Eleven tests were performed with red blood cells from C3H mice with a transplanted tumor. The findings revealed a variable behavior of hemolysis. Four tests showed a strong (3+ to 4+) hemolysis similar to that of red blood cells from normal C3H mice, while four additional tests showed the same degree of hemolysis, but in two- to fourfold higher serum dilutions. No hemolysis occurred in the three remaining instances.

The results of control tests with normal rabbit serum did not differ essentially from those obtained with antitumor rabbit serum.

Hemolysis with Tumor Suspension

Experiments were also carried out to study the hemolytic behavior of mouse erythrocytes with tumor suspensions. Gross (4), who observed that mouse erythrocytes were hemolyzed by various mouse tumors including the mammary cancer, did not differentiate between the mouse erythrocytes of different strains. It was of particular interest, therefore, to study the hemolytic effect of the mammary cancer on those mouse red blood cells which did not hemolyze with saline or rabbit serum.

A 10 per cent tumor saline suspension was freshly prepared in the cold either immediately after the death of the animal or from a tumor kept at −20° C. for a variable length of time (1–168 days). The suspension was either filtered through a silk mesh with 200 openings per square inch, or it was centrifuged for 5 minutes at 3,250 r.p.m. and the supernatant used. The tumor suspensions were tested undiluted and in dilutions of 1:2 and 1:4. Hemolysis experiments were performed in the same manner as described above. Readings were taken after various periods. As the hemolytic effect of the tumor proved to be an early one, only the findings within a period of 3–24 hours were found to be valuable. The hemolytic effect of the tumor on the C57 erythrocytes which did not show hemolysis with saline or rabbit serum was taken as the index of the hemolytic potency of the tumor.

The findings of these tests were as follows: In 37 out of 43 instances C57 red blood cells were hemolyzed by freshly prepared suspensions of spontaneous tumors and transplants up to the thirteenth passage. C3H erythrocytes were also hemolyzed, even to a more marked degree, and with tumor suspensions diluted 1:2 and 1:4, which did not affect the C57 erythrocytes. Red blood cells from hybrids and from foster-nursed C57 mice, and those from pregnant mice, tested in a few experiments, showed minimal (± to 1 plus) hemolysis.

Results different from those obtained with erythrocytes from nontumorous C3H mice were observed in 37 tests in which the red blood cells were obtained from C3H mice with spontaneous tumors. Only in 16 experiments was hemolysis similar to that of erythrocytes from C57 and from nontumorous C3H mice. In seven tests the erythrocytes from the C3H mice with spontaneous tumors showed less hemolysis and in fourteen tests no hemolysis at all.

The hemolytic potency of the tumor suspensions was impaired or completely destroyed by incubation at 37° C. or by standing at room temperature for a few hours, and also by freezing of the suspensions in a dry ice-alcohol mixture or at −20° C. for varying lengths of time.

It is noteworthy that suspensions of a transplanted tumor after 16–19 mouse passages did not display a hemolytic effect. These nonhemolyzing tumors showed rapid growth when transplanted into mice.

Effect of Antitumor Rabbit Serum on the Hemolysis of Mouse Erythrocytes by Tumor Suspension

Two methods were used to study the effect of antitumor rabbit serum on the hemolysis of mouse erythrocytes by tumor suspensions:

Neutralization of the tumor by antitumor rabbit serum.—Tumor suspension was mixed in equal

amounts with serial dilutions of antitumor rabbit serum. Normal rabbit serum was employed as the control. The mixtures were tested for hemolysis of mouse erythrocytes either immediately or after incubation, at temperatures of 4° C. or 37° C. over periods ranging from 15 minutes to 2 hours.

In 8 out of 25 tests the hemolytic effect of the tumor on C57 red blood cells was inhibited by antitumor rabbit serum but not by normal rabbit serum. Hemolysis of the C3H erythrocytes was not inhibited. These results were not obtained when tumors of low grade hemolytic potency were used or when the incubation time lasted for 1–2 hours. In these instances the hemolytic effect readings were taken. In control tests, 0.2 cc. of saline instead of tumor suspension was added to the sensitized red blood cells, and the tests were incubated as above. Readings were taken at 20–24 hours and at 40–45 hours.

The findings of these tests were consistent. It was observed that the hemolytic effect of the tumor suspension on C3H and C57 erythrocytes was inhibited after sensitization of the red blood cells with antitumor rabbit serum and with normal rabbit serum. However, the inhibition was much stronger after sensitization with antitumor rabbit serum than with normal rabbit serum. The difference in titer of inhibition is seen in Table 1. The effect of the tumor suspension on sensitized

TABLE 1

INHIBITION OF THE HEMOLYTIC EFFECT OF TUMOR SUSPENSION ON MOUSE ERYTHROCYTES SENSITIZED WITH ANTITUMOR RABBIT SERUM AND NORMAL RABBIT SERUM

(8 Tests*)

	SENSITIZED WITH:															
RED BLOOD CELL	Antitumor rabbit serum no. 70	Normal rabbit serum	Antitumor rabbit serum no. 69	Normal rabbit serum	Antitumor rabbit serum no. 76	Normal rabbit serum	Antitumor rabbit serum no. 75	Normal rabbit serum	Antitumor rabbit serum no. 72	Normal rabbit serum	Antitumor rabbit serum No. 77	Normal rabbit serum	Antitumor rabbit serum no. 75†	Normal rabbit serum	Antitumor rabbit serum no. 72†	Normal rabbit serum
C3H	128	32	64	16	32	8	32	16	16	4	32	8	32	4	32	8
C57	128	4	64	8	64	16	32	8	64	8	32	16	32	8	64	8
C3H with spontaneous tumor	128	16	128	16	64	16	64	16	64	8	64	8	32	8	64	8

* Figures denote highest dilution of serum inhibiting hemolysis.
† Sera kept at −20° C.

of the tumor on C57 red blood cells was inhibited by antitumor rabbit serum and also by normal rabbit serum. Hemolysis of the C3H erythrocytes occurred in some of the tests but not in others.

Sensitization of mouse erythrocytes with antitumor rabbit serum.—Two-tenths cc. of red blood cell suspension was added to 0.2 cc. of twofold serial dilutions of antitumor rabbit serum and to corresponding dilutions of normal rabbit serum, respectively. The mixtures were incubated for 60 minutes at 37° C. and then for 60 minutes at 4° C. Afterwards, they were centrifuged for 4 minutes at 2,000 r.p.m., and the supernatant sera were discarded. The red blood cells were taken up in 2 cc. of cold sterile saline, centrifuged again for 4 minutes at 2,000 r.p.m., and freed from the saline. Two-tenths cc. of freshly prepared tumor suspension was added immediately to the red blood cell sediments in the cold. These mixtures were incubated for 60 minutes in the water bath at 37° C. and placed in the refrigerator until final red blood cells from C3H mice with spontaneous tumor was similar to that upon erythrocytes from nontumorous mice. In the saline control series no hemolysis was noted after 24 hours.

DISCUSSION

This study revealed a difference in the hemolytic behavior of erythrocytes from a tumor-susceptible (C3H) and a tumor-resistant (C57) mouse strain and also a difference in the hemolytic behavior of erythrocytes from mice with and without mammary cancer. The incidence of hemolysis of the erythrocytes from tumor-susceptible C3H mice was high. These animals are known to be carriers of the Bittner milk agent (3). The red blood cells from tumor-resistant C57 black mice which normally do not possess the milk agent did not show hemolysis, with one exception of a spontaneous breast carcinoma. When, however, C57 mice acquired the milk agent through foster-nursing by a C3H mother, the erythrocytes ex-

With What!!

hibited hemolysis. On the other hand, erythrocytes from C3H-C57 hybrids, which looked like C3H mice and were nursed by C57 mothers, showed less hemolysis than those from pure strain C3H mice.

The question arises as to what causes the hemolysis of the erythrocytes in these experiments. From the data it seems suggestive that the milk agent plays a role in the hemolysis of the erythrocytes, either by its presence within the red blood cells or by an extracellular effect on the erythrocytes. However, this needs further investigation—especially since the incidence and amount of the milk agent in the blood cells and in the serum of nontumorous individuals from cancerous stocks are not fully established. From observations of inoculation experiments, Bittner (2, 3) stated that the agent was active in both suspensions of blood cells and the serum. Hummel and Little (5) found that blood, except from females with spontaneous mammary cancers, is a relatively poor source of the mammary tumor agent.

A striking difference between the hemolytic behavior of red blood cells from C3H mice bearing a spontaneous breast tumor and those from nontumorous individuals was found. This observation indicates that the development of the spontaneous breast tumor of the mouse is associated with changes of reactivity of the red blood cells. It cannot be stated at present if the same holds true for the transplanted tumor, because of the limited number of experiments.

A hemolysin present in tumor suspensions from mammary cancer of mice also acts on mouse erythrocytes. These findings confirm the observation of Gross (4) but point to a broader significance, since a hemolytic factor has been demonstrated in the erythrocytes of the tumor-susceptible C3H mice. The relation of the hemolysin of tumor suspensions and the red blood cell hemolysin has not yet been established.

It is of interest that the hemolysin of tumor suspensions has certain features in common with the hemolysin associated with the mumps virus (7) and also with that of the Newcastle disease virus (6): (*a*) it is similarly affected by heating to 50° C., by standing at room temperature, and by freezing; (*b*) like the Newcastle disease virus hemolysin, it reveals a discrepancy between hemolytic potency *in vitro* and invasion power in mice; (*c*) it can be inhibited by specific immune serum. However, the hemolysin observed in tumor suspensions has at least one characteristic of its own: it acts on the red blood cells of the species in which it causes the disease.

SUMMARY

A hemolytic factor was observed in the red blood cells of a tumor-susceptible (C3H) mouse strain but was not present in those of a tumor-resistant (C57 black) strain.

The red blood cells from hybrids derived from C3H fathers and C57 mothers, nursed by these mothers, and those from C57 mice fostered by C3H mothers showed hemolysis which was less marked than that of erythrocytes from C3H mice.

The red blood cells of C3H mice bearing spontaneous breast tumors reacted differently from those of nontumorous C3H individuals. They showed only limited hemolysis. The red blood cells of C3H mice with transplanted breast tumors did not show a persistent pattern of hemolysis.

A hemolysin present in suspensions of the mammary cancer of mice acted almost equally on red blood cells of C57 and of normal C3H mice. It affected erythrocytes from C3H mice with spontaneous tumor to a lesser degree or not at all.

The hemolytic effect of suspensions from mammary cancer of mice on mouse erythrocytes was more inhibited after sensitization of the erythrocytes with antitumor rabbit serum than with normal rabbit serum.

A possible relation of the hemolytic factor of the erythrocytes with the Bittner milk agent and with the hemolysin present in suspensions of the mammary cancer of mice was discussed.

REFERENCES

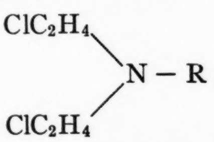
1. Adelsberger, L. Differences in Immunologic Reactions of Red Blood Cells of a Tumor-susceptible (C3H) and a Tumor-resistant (C57) Mouse Strain. Cancer Research, **11**: 653–57, 1951.
2. Bittner, J. J. Characteristics of the Mammary Tumor Milk Agent in Serial Dilution and Blood Studies. Proc. Soc. Exper. Biol. & Med., **59**: 43–44, 1945.
3. ———. The Mammary Tumor Milk Agent. Ann. New York Acad. Sc., **49**: 69–73, 1947.
4. Gross, L. Destructive Action of Mouse and Rat Tumor Extracts on Red Blood Cells *in vitro*. J. Immunol., **59**: 173–88, 1948.
5. Hummel, K. P., and Little, C. C. Studies on the Mouse Mammary Tumor Agent. I. The Agent in Blood and Other Tissues in Relation to the Physiologic or Endocrine State of the Donor. Cancer Research, **9**: 129–34, 1949.
6. Kilham, L. A Newcastle Disease Virus (NDV) Hemolysin. Proc. Soc. Exper. Biol. & Med., **71**: 63–66, 1949.
7. Morgan, H. R.; Enders, J. F.; and Wagley, P. F. A Hemolysin Associated with the Mumps Virus. J. Exper. Med., **88**: 503–14, 1948.

The Action of Some Chemical Growth Inhibitors on Healthy and Tumor Tissue of Plants

R. S. de Ropp

(*New York Botanical Garden, New York, N.Y.*†)

Various chemical agents have been found to be valuable in the treatment of cancer because of the inhibitory action they exert on the growth of malignant neoplasms. Some of the nitrogen mustards with the general formula



exert a favorable effect on the course of leukemia (4). Some analogues of pteroylglutamic acid (PGA) have a destructive or inhibitory action on a number of neoplastic growths in rats and mice (7). Among variously substituted purines and pyrimidines which have been tested, 2,6-diamino purine had a marked inhibitory effect on the growth of rat sarcoma (7), and 8-azaguanine (guanazolo) proved capable of inhibiting the growth of transplanted adenocarcinoma (E 0771) in mice (5).

Very little is known about the action of these substances on the growth of either normal or tumor tissues of plants. Several analogues of pteroylglutamic acid have been applied to carrot fragments inoculated with crown-gall bacteria and have been shown to be capable of inhibiting the growth of tumors initiated by this agent (1). It was noted that one of the nitrogen mustards inhibited mitosis in onion root tips and produced chromosomal disturbances (6). These compounds were also found capable of inducing nutritional deficiencies in *Saccharomyces cerevisiae* (8).

Experiments were undertaken to extend our knowledge of the action of these and a number of other growth inhibitors on the growth of healthy and crown-gall tumor tissues of plants. The research was divided into four parts. First, a survey was conducted with crown-gall tumors as test objects, to determine how many out of 53 different substances had the capacity to inhibit tumor growth when applied at a standard concentration. Second, compounds found to be active by this test were retested over a suitable range of concentrations to determine their degree of activity. Third, *in vitro* tests were performed with bacteria-free crown-gall tissues and healthy tissues to determine whether differences existed in the sensitivity of these tissues to the growth inhibitors. Fourth, where growth inhibition was considered to be due to the displacement of an essential metabolite, experiments were conducted to determine whether the inhibition could be reversed by the metabolite in question. Histological studies were also performed to determine the effects of the inhibitory substances on the structure of the tissues.

MATERIALS AND METHODS

Discs of carrot tissue were used as test objects in the first survey, and some results obtained with this material have been described (1). Because the response of this tissue to the crown-gall organism varied greatly, it was abandoned in favor of stem fragments of the garden chrysanthemum, variety Golden Treasure. The stem fragments were removed from the apical internodes after these had been surface-sterilized by immersion for 2 minutes in 10 per cent Clorox. Each fragment measured about 4 mm. It was freed of Clorox by several rinses in sterile water and was transferred to 10 × 70-mm. agglutination tubes containing 3 ml. of 1 per cent agar with 2 per cent sucrose and White's mineral solution. The fragment was placed on the agar in an inverted position. One loopful of a suspension of a 24-hour culture of *Agrobacterium tumefaciens*, strain BP, containing about one billion organisms per milliliter was placed on the upper cut surface of the stem fragment. Tumor growth on the inoculated surface became macroscopically visible 3 days later. To this surface the material to be tested was applied by means of a standardized loop of nichrome wire

* This work was done in part on a grant-in-aid from the American Cancer Society on recommendation by the Committee on Growth of the National Research Council.

† Present address: Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

Received for publication February 26, 1951.

delivering approximately 0.008 ml. The materials were dissolved in water, and, in all instances where solubility permitted, a concentration of 1 mg/ml was employed. About 0.008 mg. of the substance was thus applied to the tumor tissue. Three and 6 days after application of the material, the number of fragments with and without tumors was counted, and notes were made of the appearance of the tumor tissue. Ten fragments were used for each substance tested.

Those substances shown by the preliminary survey to have an inhibitory action on the growth of tumor tissue on chrysanthemum were subjected to further tests. Bacteria-free crown-gall tissue of sunflower (strain P II), aseptically removed sunflower embryos (var. Russian Giant), and fragments of excised tomato roots of the strain isolated by Robbins and Schmidt (9) were grown in nutrient media containing different concentrations of the active compounds. The rate of increase of fresh and dry weight of these tissues in the presence of these substances was determined, and any morphological effects of the compounds were noted.

Information regarding the cytological action of the active compounds was sought by growing onion roots for 24 and 48 hours in solutions of the materials. Roots treated in this way were examined in squash preparations made according to the method of Warmke, aceto orcein being used instead of aceto carmine. Material was also imbedded, sectioned at 15 μ, and stained with hematoxylin.

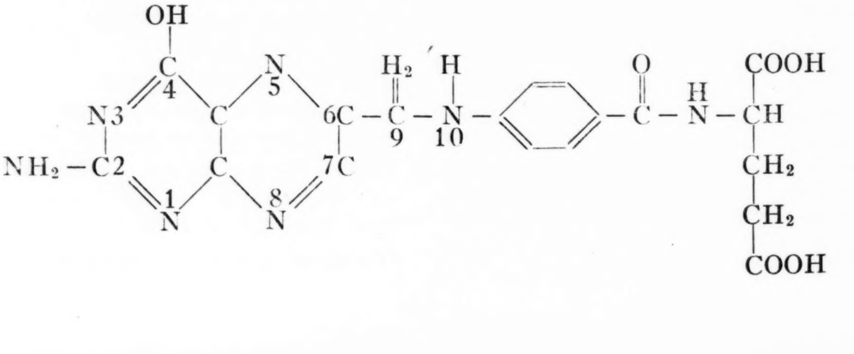
The materials tested were obtained from these sources: folic acid, its analogues, guanazolo and aureomycin, from Lederle Laboratories, Pearl River, New York; nitrogen mustards and purine and pyrimidine derivatives, neopyrithiamine and streptomycin, from Merck & Company; polymyxin hydrochloride and six sulfonamides from the American Cyanamid Company; meta-, para- and ortho-chlorophenoxy acetic acid from Dr. P. W. Zimmerman of the Boyce Thompson Institute; tyrocidin, tyrothricin, and gramicidin from Wallerstein Company; cortisone from the committee on the investigation of cortisone of the National Academy of Sciences.

RESULTS

Initial survey.—Fifty-three compounds were tested, of which 39 had no inhibiting action on the growth of crown-gall tumors on chrysanthemum stem fragments at a concentration of 1 mg/ml. The inactive compounds were as follows: *Sulfonamides*, sulfanilamide, sulfacetamide, sulfadiazine, sulfaguanine, sulfathiazole, sulfapyridine, Darvisul; *antibiotic substances*, penicillin G., streptomycin, aureomycin, tyrocidin, polymyxin, tyrothricin; *purine and pyrimidine derivatives*, 2,6-dihydroxy-4,5-diamino pyrimidine sulfate, 2,5-diamino-4,6-dihydroxypyrimidine sulfate, hexahydro-1,3,5-tris-isobutyryl-*s*-triazine, 2,6-diamino purine; *vitamins and analogues*,[1] folic acid, Diopterin, Teropterin, Gamopterin, Triglutam, Teralpherin, Amino-Treofol, Metfol-B, Anfol-A, Anfol-R, desoxypyridoxine, neopyrithiamine, xanthopterin, 4-amino-2-hydroxy-benzoic acid; *plant growth regulators*, indol-3-acetic acid, indole butyric acid, ortho-, para- and meta-chlorophenoxy acetic acid; *nitrogen mustards*, β-chloroethylamine, *n*-butylbis(β-fluoroethyl)amine. Data on the sixteen active compounds are shown in Table 1.

The compounds with the most powerful inhibitory action on the growth of these plant tumors belonged to the group of analogues of folic acid, the action of which on animal neoplasms has already been mentioned. These active compounds had in common the replacement with an amino group of the hydroxy group in the 4-position of the pteroylglutamic acid molecule, the structure of which is shown at the bottom of the page.

[1] Amino-Treofol (4-Aminopteroyl-DL-threonine)
Anfol-A (Pteroylaspartic acid)
Anfol-R (Pteroyl-DL-aspartic acid)
Diopterin (Pteroyl-α-glutamylglutamic acid)
Gamopterin (Pteroyl-γ-glutamylglutamic acid)
Metfol-B (N^{10}-Methylpteroic acid)
Teralpherin (Pteroyl-α-glutamyl-α-glutamylglutamic acid)
Teropterin (Pteroyl-γ-glutamyl-γ-glutamylglutamic acid)
Triglutam (p-Aminobenzoyl-γ-glutamyl-γ-glutamylglutamic acid)



The introduction of a methyl group in the 9- or N^{10}-position increased the activity of these substances. At 1 mg/ml most of these compounds not only inhibited the growth of tumor tissue but also caused extensive necrosis of the stem fragment. The full extent of this action could be seen in fixed and stained material. Cells in the affected area were collapsed, and their nuclei were shrunken. Lower concentrations of the substances had progressively more localized effects. The most obvious only reduced the amount of tumor growth without inhibiting it completely.

The inhibition of tumor growth produced by naphthalene acetic acid and 2,4-dichlorophenoxyacetic acid was not persistent. 8-Azaguanine caused persistent inhibition of tumor growth. Cortisone at 0.1 mg/ml caused partial inhibition.

Comparative activity.—The six active analogues of PGA shown in Table 1 were added to nutrient media to give concentrations of 1,000, 100, 10, 1,

TABLE 1

TUMOR GROWTH ON CHRYSANTHEMUM STEM FRAGMENTS 3 AND 6 DAYS AFTER APPLICATION OF GROWTH INHIBITORS

(++ = growth as great as controls; + = growth less than half controls; 0 = no growth)
(means of 10 estimates)

SUBSTANCE	CONCENTRATION (MG/ML)	TUMOR GROWTH 3 days	TUMOR GROWTH 6 days
4-Amino-N^{10}-methyl-pteroylglutamic acid	1.0	0	0
(A-Methopterin)	0.1	0	0
	0.01	0	0
4-Amino-9-methyl-pteroylglutamic acid	1.0	0	0
(A-Ninopterin)	0.1	0	0
	0.001	0	+
4-Aminopteroylglutamic acid	1.0	0	0
(Aminopterin)	0.1	0	0
	0.01	0	+
4-Aminopteroylaspartic acid	1.0	0	0
(Amino-Anfol)	0.1	0	+
	0.01	+	++
4-Aminopteroyl-γ-glutamyl-γ-glutamylglutamic acid	1.0	0	0
(Amino-Teropterin)	0.1	0	0
	0.01	0	++
4-Amino-9, N^{10}-dimethylpteroylglutamic acid	1.0	0	0
(A-Denopterin)	0.1	0	0
	0.01	0	+
Methylbis(β-chloroethyl)amine	1.0	0	0
	0.1	0	+
1,3-bis-(bis-[2-chloroethyl]amino)-propane 2 HCl	0.1	+	+
Methylbis(β-bromoethyl)amine HBr	0.1	0	0
N,N-(β-chloroethyl)aniline	1.0	+	+
Tris(2-chloro-*n*-propyl)amine, $HCl \cdot \frac{1}{2}H_2O$	1.0	+	+
Ethylenediamine tetraacetic acid	1.0	+	+
Naphthaleneacetic acid	1.0	0	+
2,4-Dichlorophenoxyacetic acid	1.0	0	+
8-Azaguanine (guanazolo)	1.0	0	0
Cortisone	0.1	0	+

characteristic of this sectioned material was the absence of mitoses from the tissues which had come into contact with the active compounds.

The nitrogen mustards as a group exerted a much more feeble inhibiting action on the growth of tumor tissue on these stem fragments than did the folic acid analogues. Methylbis(β-chloroethyl)-amine proved the most active substance of the group. At a concentration of 1 mg/ml it caused blackening and necrosis of most of the stem fragment. At 0.1 mg/ml its growth-inhibiting action was confined to the tumor tissue. Growth inhibition was not complete at this concentration. The bromine analogue of this substance was about equally active, but the other nitrogen mustards

0.1, 0.01, and 0.001 mg/l. Three different media were used. For bacteria-free crown-gall tissue a liquid medium containing 2 per cent sucrose and White's mineral solution was employed. It was placed in culture tubes equipped with ridges which, when placed on their sides, exposed a large area of the culture medium to the air. These tubes held 5 ml. of medium each. The fragments of tumor tissue when placed within them projected partly into the air.

For excised tomato roots the medium described by Robbins and Schmidt (9) was placed in 20-ml. amounts in tubes to which the root fragments were then added. The excised sunflower embryos were cultured on a medium containing 1 per cent

agar, 2 per cent sucrose, and White's mineral solution distributed to tubes in 20-ml. amounts. The average fresh weight of tumor tissue added to each tube of nutrient was 75 mg. The fresh weight of the fragment of excised tomato root was only 1 mg. and that of the sunflower embryo 55 mg. The fragment of tomato root was thus exposed to about 250 times as much active material per milligram fresh weight as was the tumor tissue.

The tumor tissue and tomato roots were cultured in diffuse light for 4 weeks and the sunflower embryos in complete darkness for 3. At the end of this time they were removed and their fresh and dry weights determined. Ten replicates were used for each treatment. Concentrations of 10 mg. or more per liter of the substances tested completely inhibited the growth of all these tissues. The amount of growth inhibition brought about by concentrations from 100 to 1 μg/l is shown in Table 2.

The three compounds A-Denopterin, A-Methopterin and A-Ninopterin proved, on the whole, to have greater inhibitory power than had Amino-Anfol, Amino-Teropterin, and Aminopterin. Of the three tissues tested excised tomato roots proved most sensitive to the inhibitory action of these folic acid analogues, their growth being completely inhibited by as little as 1 μg/l of A-Denopterin. Crown-gall tumor tissue was least inhibited. Root growth on the sunflower embryos was much more strongly inhibited than was stem growth. Even in the presence of 100 μg/l of all the active compounds, the cotyledons of these embryos enlarged, although root growth was completely checked.

To determine whether the presence of light affected the action of these compounds, sunflower embryos were grown in continuous light in the presence of 1,000, 100, 10, and 1 μg/l of A-Methopterin. The appearance of these embryos after 3 weeks in culture on agar containing this substance is shown in Figure 1. Root growth was strongly inhibited by as little as 10 μg/l of the compound. Some elongation of the hypocotyl and expansion and greening of the cotyledons took place even on the highest concentration of A-Methopterin by which root growth was completely inhibited.

The comparative action of one nitrogen mustard (methylbis[β-chloroethyl]amine) was tested on these three kinds of tissues. Because this compound breaks down rapidly in solution, it was not incorporated into the media in which the tissues were grown. Instead, the tissue fragments were placed in empty sterile flasks, to which the freshly prepared nitrogen mustard solution was added. Tissues were left in contact with the substance for 1 hour, then were washed and transferred to nutrient media as described above. Sunflower embryos were allowed to germinate for 24 hours on 1 per cent agar before being exposed to the nitrogen mustard.

The amount of growth made by these tissues after 1 hour's contact with solution containing 1,000, 100, 10, and 1 mg/l of the nitrogen mustard is shown in Table 3. Growth of the excised tomato roots was most strongly inhibited by this exposure to the nitrogen mustard. Exposure to the two highest concentrations caused complete inhibition of the growth of tumor tissue. The sunflower embryos, cultured in this instance in light, were least inhibited. Their root growth was much more severely curtailed than was their top growth. Some of the plants developed abnormally, with leaves growing in rosettes, but these abnormal plants all died after about 6 weeks.

The action of 8-azaguanine on these three different tissues was tested at 10, 1, and 0.1 μg/l. At all these concentrations it completely inhibited the growth of excised tomato roots and reduced the growth of the other tissues to a significant but smaller extent.

The action of cortisone was tested on tumor tissue alone. One mg/ml of this compound completely inhibited the growth of tumor tissue *in vitro*. As soon as fragments were removed to a nutrient freed from the cortisone, they once more grew at the same rate as did the controls.

Recovery from and reversal of inhibition.—Fragments of bacteria-free crown-gall tumor tissue were grown for 4 weeks in liquid nutrient containing 100, 10, 1, and 0.1 mg/l A-Methopterin. At the end of this time the fragments were washed in sterile distilled water, weighed individually, and transferred to nutrient agar containing 1 mg/l PGA. On this medium they were cultured for an additional 4 weeks, at the end of which time their fresh weights were again determined. The inhibition of growth induced by the A-Methopterin was not reversed by the PGA. The fragments that had been exposed to the lowest concentration of A-Methopterin made some additional growth, but this growth was only about one-fifth of that made by the controls.

The growth of fragments of tumor tissue in liquid medium containing mixtures of A-Methopterin and PGA was next studied. One concentration of A-Methopterin (10 μg/l) was combined with 4 concentrations of PGA (10, 1, 0.1, and 0.01 mg/l). Media were also prepared containing PGA alone, A-Methopterin alone, and neither substance. None of the concentrations of PGA em-

ployed was capable of antagonizing the inhibitory action of the A-Methopterin. By itself the PGA had an inhibitory action on the growth of this tissue at a concentration of 100 mg/l, but no action at lower concentrations.

It has been shown (2) that reversal of Aminopterin-induced inhibition of Sarcoma 180 by PGA was only complete when injections of the latter were begun 1 day prior to the administration of the Aminopterin. For this reason the above experiment was repeated in part using a nutrient medium containing 10 mg/l PGA. The fragments of tumor tissue were cultured for 3 days in this medium, to which A-Methopterin was then added to give a final concentration of 10 μg/l. After 4 weeks of growth in this medium the fragments were weighed. Despite the prior addition of the PGA, no significant antagonism of the A-Methopterin–induced inhibition of the growth of these tissue fragments could be detected. It was not found possible to reverse the inhibitory action of 8-azaguanine by the addition to the medium of 0.1 mg/ml of guanine.

Histological observations.—Sections were prepared both of chrysanthemum stem tissue and of onion roots treated with various folic acid analogues and nitrogen mustards. Onion roots grown in 1 and 0.1 mg/ml of A-Methopterin were devoid of mitoses after 24 hours but developed them after 48 hours. No clear evidence of abnormal cell division was obtained. The stimulation of cell division observed by Jakowska *et al.* (3) in onion roots treated with 0.001 mg. per cent Aminopterin was not observed in any roots used in this work.

The nitrogen mustard, methylbis(β-chloroethyl)amine, apparently killed the onion roots exposed to it in concentrations of 1 and 0.1 mg/ml. Roots growing in 0.01 mg/ml of this substance

TABLE 2

GROWTH (4 WEEKS) OF SUNFLOWER TUMOR TISSUE AND TOMATO ROOTS AND GROWTH 3 WEEKS OF SUNFLOWER EMBRYOS ON MEDIA CONTAINING ANALOGUES OF FOLIC ACID
(means of 10 estimates)

Substance	Conc. (μg/l)	Tumor tissue Fresh wt. (mg.)	Tumor tissue Dry wt. (mg.)	Tomato roots Fresh wt. (mg.)	Tomato roots Dry wt. (mg.)	Sunflower embryos Fresh wt. (mg.)	Sunflower embryos Dry wt. (mg.)
A-Denopterin	100	86	6	1.0	0.06	310	4.4
	10	129	12	1.0	0.05	524	5.4
	1	191	14	1.0	0.07	1,271	7.9
A-Methopterin	100	108	8	1.2	0.08	244	5.3
	10	143	10	1.4	0.07	608	5.9
	1	195	14	4.5	0.22	1,202	9.5
A-Ninopterin	100	93	7	1.5	0.08	277	5.3
	10	113	9	1.0	0.07	516	5.8
	1	190	17	7.4	0.50	1,202	8.2
Amino-Anfol	100	205	14	1.4	0.09	502	6.0
	10	253	19	2.6	0.13	913	6.2
	1	254	19	23.8	1.66	1,435	8.8
Amino-Teropterin	100	117	9	1.0	0.07		
	10	182	14	3.5	0.27		
	1	226	16	29.6	2.13		
Aminopterin	100	98	7	0.8	0.05	459	5.5
	10	182	13	0.9	0.21	811	6.7
	1	228	16	32.8	2.30	1,584	9.5
Control	0	256	20	60.2	4.60	1,607	10.8
Initial weights		75	6	1.0	0.05	55	

TABLE 3

GROWTH MADE BY TUMOR TISSUE AND TOMATO ROOTS IN 4 WEEKS AND SUNFLOWER EMBRYOS IN 3 AFTER TREATMENT FOR 1 HOUR WITH METHYLBIS(β-CHLOROETHYL)AMINE
(means of 10 estimates)

Conc. (mg/L)	Tumor tissue Fresh wt. (mg.)	Tumor tissue Dry wt. (mg.)	Tomato roots Fresh wt. (mg.)	Tomato roots Dry wt. (mg.)	Sunflower embryos Fresh wt. Top (mg.)	Sunflower embryos Fresh wt. Root (mg.)	Sunflower embryos Dry wt. Top (mg.)	Sunflower embryos Dry wt. Root (mg.)
1,000	65	3	1.0	0.06	205	11	11	0.8
100	63	3	0.9	0.05	431	71	22	3
10	261	11	1.6	0.08	720	289	58	20
1	411	23	2.0	0.13	769	661	64	41
Control	463	23	61.3	4.75	1,203	1,103	112	69

were almost completely devoid of mitoses after 24 hours, but cell divisions again became visible after 48 hours. The abnormal divisions such as were figured by Novick and Sparrow (6) could not be demonstrated with certainty in this material. Inhibition of mitoses was thus one of the results of treatment of these roots both with the six active folic acid analogues and with the nitrogen mustard. It is not possible to be sure whether this was a direct or indirect result of the action of these compounds.

DISCUSSION

The research described in this paper was directed toward two main aims: first, to determine the degree of growth-inhibiting action possessed by a number of chemical agents; second, to discover whether any of these agents would inhibit the growth of crown-gall tumor tissue to a significantly greater extent than the growth of healthy tissue.

The work has shown that four different compounds or groups of compounds—analogues of pteroylglutamic acid, certain nitrogen mustards, 8-azaguanine, and cortisone—can exert an inhibitory effect on the growth of crown-gall tumor tissue. The strongest inhibitory action was exerted by three PGA analogues in which the 9- or N^{10}-methyl group was present in addition to the 4 amino group. These compounds exerted a measurable inhibitory action at a concentration of 1 $\mu g/l$. The nitrogen mustards, 8-azaguanine and cortisone, were less potent.

When these substances were tested on excised tomato roots or on sunflower embryos cultured *in vitro*, they were found to exert an equally powerful or more powerful growth-inhibiting action. Excised tomato roots proved particularly sensitive to the growth-inhibiting action of these compounds. Evidently none of these agents was capable of exerting a differential inhibiting action on the growth of tumorous as opposed to healthy plant tissue.

The mechanism of action of the PGA analogues remains obscure. No amount of added PGA antagonized the action of the analogue, even when the PGA was added in advance. If these analogues act in the plant by blocking enzyme systems which depend for their functioning on PGA, then it would appear that the analogues have a much greater affinity for such enzymes than has PGA itself.

SUMMARY

Fifty-three substances were applied to fragments of chrysanthemum stem 3 days after they had been inoculated with crown-gall bacteria. Sixteen of these compounds inhibited tumor formation at a concentration of 1 mg/ml. The growth-inhibiting capacities of some of these compounds on bacteria-free crown-gall tumor tissue, excised tomato roots, and sunflower embryos were compared. Six analogues of folic acid—A-Methopterin, A-Denopterin, A-Ninopterin, Amino-Teropterin, Amino-Anfol, and Aminopterin—inhibited the growth of excised tomato roots at concentrations of from 1 to 10 $\mu g/l$. Bacteria-free crown-gall tissue and sunflower embryos were slightly less sensitive to the inhibitory action of these compounds.

The nitrogen mustard, methylbis(β-chloroethyl)amine, completely inhibited the growth of tomato roots exposed for 1 hour to a 1 mg/l solution. Tumor tissue and sunflower embryos were less sensitive to the action of this agent. 8-Azaguanine completely inhibited the growth of excised tomato roots at a concentration of 0.1 mg/l. The two other tissues were also less sensitive to this agent. Cortisone in a concentration of 1 mg/ml inhibited the growth of crown-gall tissue, but growth recommenced when the tumors were transferred to a cortisone-free medium.

The inhibitory action of A-Methopterin (10 $\mu g/l$) on crown-gall tissue could not be reversed with pteroylglutamic acid. At a concentration of 100 mg/l, this substance was itself toxic to the tissues. The inhibiting action of 8-azaguanine was not reversed with guanine.

Sections of tissues treated with PGA antagonists or nitrogen mustards revealed an absence of mitoses, but no definite evidence of abnormal mitoses was obtained.

REFERENCES

1. DE ROPP, R. S. Inhibiting Action of Some Analogues of Folic Acid on the Growth of Plant Tumors. Nature, **164**: 954, 1949.
2. GOLDIN, A.; GOLDBERG, B.; ORTEGA, L. G.; and SCHOENBACH, E. B. Reversal of Aminopterin-induced Inhibition of Sarcoma 180 by Folic Acid. Cancer, **2**:857, 1949.
3. JAKOWSKA, S.; NIGRELLI, R. F.; and GOLDSMITH, E. D. Plant Growth Regulating Effects of Aminopterin. Am. J. Bot., **36**:825, 1949.
4. KARNOFSKY, D. A.; BURCHENAL, J. H.; ORMSBEE, R. A.; CORNMAN, I.; and RHOADS, C. P. Approaches to Tumor Chemotherapy. Washington, D.C.: A.A.A.S., 1947.
5. KIDDER, G. W.; DEWEY, V. C.; PARKS, R. E., JR.; and WOODSIDE, G. L. Purine Metabolism in *Tetrahymena* and Its Relation to Malignant Cells in Mice. Science, **109**: 511–14, 1949.
6. NOVICK, A., and SPARROW, A. H. The Effects of Nitrogen Mustard on Mitoses of Onion Root Tips. J. Hered., **40**:13–17, 1949.
7. SUGIURA, K., and STOCK, C. C. The Effect of Folic Acid and Antifolic Compounds on the Growth of Carcinoma, Sarcoma, Osteogenic Sarcoma, Lymphosarcoma, and Melanoma in Animals. Cancer Research, **9**:628, 1949.
8. REAUME, S. E., and TATUM, E. L. Spontaneous and Nitrogen Mustard Induced Nutritional Deficiencies in *Saccharomyces cerevisiae*. Arch. Biochem., **22**:331–38, 1949.
9. ROBBINS, W. J., and SCHMIDT, M. B. Growth of Excised Roots of the Tomato. Bot. Gaz., **99**:671–727, 1938.

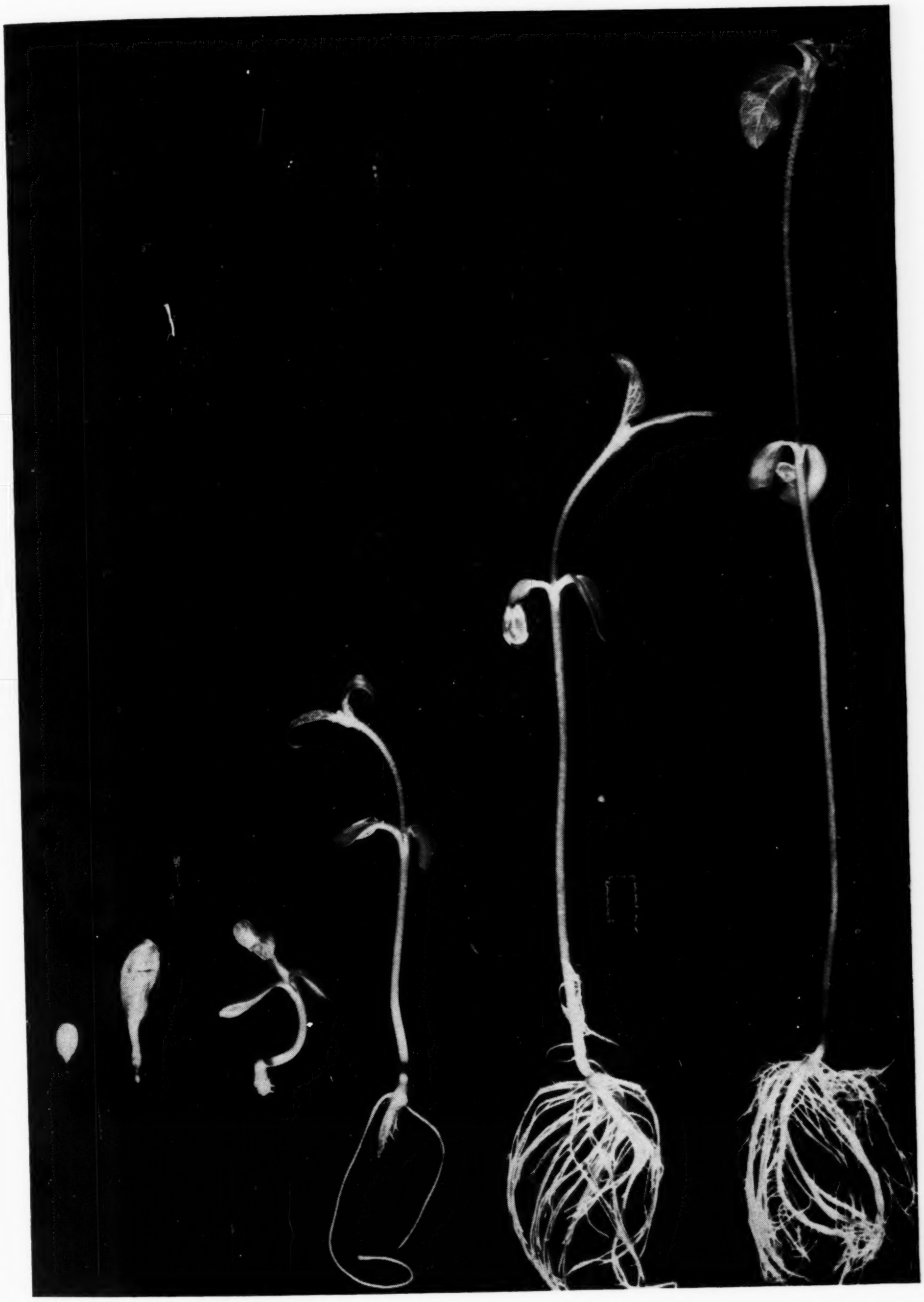


FIG. 1.—Appearance of sunflower embryos after 3 weeks' growth *in vitro* in continuous light on nutrient agar containing A-Methopterin. Left to right: original size of embryo, embryos cultured on 1,000, 100, 10, and 1$\mu g/l$ A-Methopterin, control cultured on agar devoid of A-Methopterin.

Effect of West Nile and Ilheus Viruses on Mouse Leukemias*

CHESTER M. SOUTHAM, BEVERLEY BRONSTEIN, AND LORRAINE F. WEBBER

(Section on Mouse Leukemia and the Section for Virus Studies of the Division of Experimental Chemotherapy, The Sloan-Kettering Institute for Cancer Research, New York 21, N.Y.)

An inhibitory effect of certain viruses on the growth of transplantable tumors in mice has been demonstrated by Moore and co-workers (6–8) and by Kaprowski and Norton (4). Turner and Mulliken (11) have reported a slightly increased survival rate in one strain of mouse leukemia (9417) after inoculation of vaccinia virus, but two other strains (1394 and C1498) were unaffected. Sharpless (9) has demonstrated complete eradication of leukosis in fowl following inoculation of Russian spring-summer encephalitis virus. The present communication reports the suppressive effect of West Nile and Ilheus encephalitis viruses on two strains of mouse leukemia, as determined by serial blood counts.

MATERIALS AND METHODS

Mice were of the Akm strain, obtained from Carworth Farms. They were about 45 days old and weighed 18–22 gm. at the start of each experiment. Both sexes were used, but each experimental group of 30 mice was of a single sex. Purina Laboratory Chow and water were available ad libitum.

Leukemia Ak4 has been previously described (1). Leukemia Ak 9421 arose spontaneously in a 6-month-old male mouse of Akm stock at this institute in April, 1946. Both strains are maintained in this laboratory by intraperitoneal transplantation of a suspension of cells from minced leukemic spleen into Akm mice. In the studies reported here all inocula were standardized to deliver 100,000 cells per mouse. This inoculum produced fatal leukemia of remarkably uniform course in over 95 per cent of the mice.

Most experimental groups in this study consisted of 30 mice: 10 controls, 10 to be infected with West Nile virus, and 10 with Ilheus virus.

* This investigation was carried out during tenure of a Damon Runyon Clinical Cancer Research Fellowship. The investigation was partly supported by an institutional research grant from the American Cancer Society, and additional support was obtained from the National Cancer Institute of the Public Health Service.

Received for publication March 8, 1951.

Blood cells were counted by standard clinical technics. Blood was obtained by nicking one of the tail veins with a razor blade. For ½ hour prior to taking blood counts, the mice were warmed under an electric lamp for several minutes to insure vasodilation and free flow of blood. Blood counts were usually done 3 times per week in the early stages of leukemia and daily after the leukocyte counts started to rise. Leukocyte counts were done on all mice. Erythrocyte counts and differentials were done on three randomly selected mice from each group of ten. All data in the charts and tables are arithmetic means. Since differential counts were not done on all mice, the sum of the averaged differential counts is not identical with the averaged total leukocyte counts. Obviously, the blood counts representing the last stages of leukemia represent only the few animals surviving to that time. The control curves in Charts 3–6 represent the combined data from controls of all experiments with the indicated strain of leukemia. Control data are tabulated separately for each experiment in the table.

West Nile virus (10) and Ilheus virus (5) were obtained from Dr. Hilary Kaprowski of Lederle Laboratories and maintained by intracerebral inoculation of CFW (Carworth Farms) Swiss mice. In all experiments here reported a uniform stock of either virus was utilized, prepared by emulsifying about 50 infected mouse brains in 0.85 per cent sterile saline to give a 20 per cent suspension. Suspensions were centrifuged for 10 minutes at 2,000 r.p.m. to remove large particles and were then sealed in ampoules in 5-cc. lots and maintained at about $-76°$ C. until immediately before use. Both virus preparations after freezing and thawing had an intracerebral titer of approximately 10^{-5}.

In the experimental studies the viruses were inoculated intraperitoneally in a dose of 0.5 cc. of the 20 per cent mouse brain preparations. This dosage is approximately an LD_{95} for West Nile virus and an LD_{60} for Ilheus virus, as titered intraperitoneally in normal Akm or CFW mice.

The interval between inoculation with leukemia and inoculation with virus was varied in the several experimental groups in order to observe differences in effect of virus infection initiated at various stages of the leukemic process.

Dead mice were autopsied. Wet weights of liver and spleen were recorded but are not tabulated in this presentation. If leukemia was not grossly apparent, histologic sections of liver and spleen were examined. Four mice in which leukemia was not found were discarded from the study.

Leukemic tissues were examined for the presence of virus, when indicated, by preparation of 10 per cent tissue suspensions in sterile saline and inoculation intracerebrally into CFW (leukemia resistant) mice. Virus titers are expressed as the logarithm of the greatest dilution causing death by encephalitis.

RESULTS

Effect of viruses on normal Akm mice.—Nonleukemic Akm mice infected with West Nile virus appeared well until the third day, when some animals exhibited paralysis of the hind quarters and one died. Three days later eight of the ten mice were dead, and all were dead by the ninth day after inoculation.

Of ten mice inoculated with Ilheus virus, only six succumbed to infection. In these six, hind quarter paralysis appeared about the sixth or seventh day. One mouse died on the fourth day, and the others died between 9 and 13 days after inoculation. Only the six fatally infected mice were considered in the study of hematologic effects.

Both viruses caused slight leukopenia and lymphopenia by the seventh day, as compared to the uninjected control group. Granulocytes and erythrocytes showed no variation attributable to virus. Liver and spleen weights were within normal limits at time of death.

Course of untreated Ak4 leukemia.—Ak4 leukemia, as used in this study, caused death in 6–14 days in nearly 100 per cent of inoculated Akm mice. Mean survival time was about 8 days. For about 2 days before death there was ruffling of the fur and marked inanition and paresis. Terminally, respiration was usually slow and gasping. On autopsy the striking gross findings were enlargement of axillary and inguinal nodes to about 1 mm. in diameter and marked enlargement of liver and spleen. The leukemic livers were paler than normal or were spotted with pale areas if the leukemic infiltration was not complete. Signs of hemorrhage were not usual. Histologic sections revealed widespread infiltration with leukemic cells, most consistent and most obvious in the liver.

The erythrocyte count showed considerable fluctuation but no consistent change. The course of this leukemia is so short that depression of erythropoiesis would not be expected to be reflected in the peripheral blood. The wide fluctuations observed probably represent the summation of blood loss from the tail incision and hemoconcentration due to poor water intake during the period of inanition, as well as the inaccuracies inherent in the blood counting technic. There appeared to be increased bleeding from the tail incisions in the later stage of leukemia, but thrombocytes did not appear reduced, as judged by the stained blood smears. The leukocyte count showed no abnormality for about 5 days after inoculation, but thereafter rose rapidly to levels of 100,000–400,000 cells per cubic millimeter on the day of death. The increased leukocytes were predominantly very immature cells (probably prolymphocytes and lymphoblasts), which we have arbitrarily called "blasts," but at the onset of leukocytosis there was also an increase in mature granulocytes and lymphocytes to as much as 40,000 cells per cubic millimeter. Terminally, granulocytes and mature lymphocytes disappeared.

Effect of normal brain injections on Ak4 leukemia.—One milliliter of a 20 per cent normal (CFW) mouse brain suspension was injected intraperitoneally into each of ten mice on the second day after inoculation with Ak4 leukemia, and into another group on the sixth day. There were no significant differences between the blood counts of these injected groups and of their uninjected controls. Leukemic infiltration of liver and spleen, as judged by organ weight, was just as great in the injected as in the uninjected mice, and survival times were slightly shorter. Thus, it appears that any hematologic effects observed in virus-treated mice must be attributed to the virus and not to the brain tissue.

Effect of West Nile virus on Ak4 leukemia.—West Nile virus was inoculated, in several experiments, 4 hours, 2 days, 5 days, 6 days, and 7 days after inoculation of leukemia. In each experiment leukocyte counts remained at normal or near-normal levels for 2–3 days after the blood had become leukemic in the untreated controls. There was no depression of granulocytes and only moderate depression of lymphocytes below normal levels during this period. The effect was a selective inhibition of leukemic blast cell proliferation. Since the virus usually kills in 5–9 days, studies of leukemia beyond the tenth day were rarely possible. In those few mice that did survive to the twelfth day, leukocytes and blast counts

became elevated. This suggests that the anti-leukemic effect of the virus is merely suppressive, rather than "curative" as in fowl leukosis (9). Since it is impractical to publish complete data of these studies, those data which best illustrate the anti-leukemic effect have been chosen for presentation (Table 1 and Chart 1).

Inhibition of leukemic cell proliferation in the virus-infected mice was also evidenced by the absence of hepatosplenomegaly and adenopathy in the treated mice. Weights of liver and spleen were normal at time of death in the virus-treated mice and were 50–100 per cent above normal in uninjected mice dying of leukemia. This effect has also been observed by Moore[1] in Ak4 leukemic mice infected with Russian spring-summer encephalitis virus.

Effect of Ilheus virus on Ak4 leukemia.—The results seen with Ilheus virus in Ak4 leukemia paralleled those seen with West Nile virus. Hematologic and survival data from those studies which best demonstrated the anti-leukemic effect are presented in Table 1 and Chart 2. The absence of hepatosplenomegaly in virus-infected mice was again observed. Suppression of the leukemia was usually not quite so complete as with West Nile virus, probably because the Ilheus virus inoculum did not cause infection in all animals. Uninfected animals could not be excluded from these groups, because death from virus and death from leukemia could not be distinguished, and routine testing for virus was impractical.

Localization of viruses in leukemic mice.—The site of virus proliferation was studied in Ak4 leukemic mice in two experiments.

First, since the spleen of the leukemic mouse presents accessible and numerous leukemic cells, virus titers were determined for this organ and compared with parallel titers of brain and whole blood. Virus was inoculated intraperitoneally on the fourth day of Ak4 leukemia, and one mouse infected with each virus was sacrificed 2, 4, and 7 days later for virus titration. The data demonstrate that West Nile virus was present in blood and spleen throughout the study, with spleen showing slightly higher titers (10^{-2} to 10^{-4}) than blood (10^{0} to 10^{-2}). Brain (10^{-1} suspension) contained no detectable virus at 48 hours, but on the fourth and seventh days the brain showed higher titers (10^{-4} and 10^{-5}) than blood or spleen. Ilheus virus never attained a titer over 10^{-2} in this study but was present in spleen 2 and 4 days after inoculation in higher titers (10^{-2}) than in blood or brain. Ilheus virus was not detected in the mouse sacrificed on the seventh day, probably because of failure to establish infection in the animal tested.

Second, five mice were inoculated with Ilheus virus on the fourth day of Ak4 leukemia, and after another 2 days were bled by decapitation. Heparin was added to prevent coagulation, and all blood was pooled. Erythrocytes were agglutinated by adding a few drops of a rabbit anti-mouse erythrocyte serum and were sedimented by slow centrifugation. This procedure left a large proportion of leukocytes and only a few erythrocytes in the supernatant. This supernate was decanted and then centrifuged at high speed to sediment the leukocytes. The leukocyte-rich sediment and the erythrocyte sediment were washed twice with saline, and then these preparations and the plasma were serially diluted in saline and titered for virus. No virus was detected in the erythrocytes, even in 10 per cent suspension. Virus was present in the leukocytes in a titer of 10^{-4}. Virus was present in the plasma at a titer of approximately 10^{-2}.

These two experiments indicate that the tested viruses invade leukocytes, presumably the leukemic leukocytes.

Course of untreated Ak 9421 leukemia.—Ak 9421 leukemia, as used in this study, is somewhat less acute than Ak4 leukemia. Death usually occurs between the eleventh and twentieth days after inoculation. Mean survival time after inoculation is about 14 days. Gross and microscopic pathology are as described for Ak4 leukemia. Hematologic findings differ from those of Ak4 leukemia in that the total leukocyte count rarely exceeds 50,000, and this leukocytosis is due to approximately equal increase in mature lymphocytes and lymphoblasts.

Effect of West Nile virus on Ak 9421 leukemia.—When West Nile or Ilheus virus was inoculated in the early stages of development of Ak 9421 leukemia, mice died of virus encephalitis before untreated leukemic controls showed any signs of leukemia.

When West Nile virus was inoculated on the seventh day after inoculation of leukemia, the blood counts remained essentially normal for 2 days after the controls showed leukemic counts, but there was no significant difference in survival time. When virus was given 11 days after leukemia, the same suppression of leukemic leukocytosis was observed, and in this experiment the mean survival time of the virus-infected mice was slightly longer (doubtful significance) than that of the controls. In this strain of leukemia the inhibitory effect was against both mature and immature lymphoid cells (Chart 3). (Data from

[1] A. E. Moore, personal communication.

TABLE 1

Effect of West Nile and Ilheus Viruses on Ak4 Leukemia

Days after leukemia inoculation	Virus	Leukocytes*	Blasts*	Lymphocytes	Granulocytes	Survival† Av. (days)	Survival† Range (days)
Virus Inoculated 1 Day after Ak4 Leukemia							
0	None	18.2 (9)	0 (3)	17.5	4.0		
0	W. Nile	17.1 (10)	0 (3)	14.8	4.6		
0	Ilheus	22.3 (10)	0 (2)	12.2	5.2		
2	None	10.4 (9)	0 (1)	5.4	1.7		
2	W. Nile	6.0 (10)	0 (1)	4.5	5.2		
2	Ilheus	8.0 (10)	(0)				
4	None	10.3 (9)	1.6 (3)	5.9	3.6		
4	W. Nile	11.7 (10)	1.0 (2)	6.0	5.2		
4	Ilheus	26.7 (10)	5.5 (2)	10.5	10.7		
7	None	107.2 (6)	57.0 (3)	36.0	43.7		
7	W. Nile	6.3 (7)	0.4 (3)	4.7	3.9		
7	Ilheus	34.9 (10)	9.5 (3)	10.0	34.2		
9	None	(0)	(0)				
9	W. Nile	(0)	(0)				
9	Ilheus	30.4 (2)	5.5 (2)	7.2	17.7		
11	None	(0)	(0)			7.4	(6–9)
11	W. Nile	(0)	(0)			7.2	(6–8)
11	Ilheus	153.0 (1)	104.0 (1)	22.0	27.6	9.0	(8–13)
Virus Inoculated 3 Days after Ak4 Leukemia							
2	None	17.2 (8)	3.9 (3)	6.2	4.7		
2	W. Nile	10.9 (10)	2.0 (3)	5.8	3.3		
2	Ilheus	10.8 (9)	2.5 (2)	5.6	3.7		
4	None	12.6 (8)	3.0 (3)	6.9	4.4		
4	W. Nile	6.2 (10)	0.2 (2)	1.9	2.6		
4	Ilheus	8.9 (9)	0.4 (3)	3.8	3.4		
7	None	76.1 (3)	29.8 (1)	18.6	36.6		
7	W. Nile	14.0 (10)	6.0 (2)	8.0	13.4		
7	Ilheus	76.0 (8)	18.2 (3)	15.2	32.6		
9	None	208.0 (1)	184.0 (1)	9.4	14.6	7.4	(6–10)
9	W. Nile	6.3 (2)	0.5 (1)	3.5	4.2	8.2	(7–9)
9	Ilheus	141.0 (3)	73.5 (2)	18.5	25.2	8.7	(8–11)
Virus Inoculated 5 Days after Ak4 Leukemia							
5	None	14.6 (10)	5.0 (3)	5.9	6.3		
5	W. Nile	13.4 (10)	2.9 (3)	3.7	5.8		
5	Ilheus	12.8 (8)	3.2 (3)	5.3	4.9		
6	None	13.5 (10)	3.4 (3)	7.6	5.1		
6	W. Nile	7.0 (10)	1.2 (2)	1.4	4.6		
6	Ilheus	7.0 (8)	0.5 (1)	0.9	5.5		
7	None	43.9 (10)	6.2 (3)	11.9	14.5		
7	W. Nile	15.5 (10)	0.8 (3)	6.8	3.6		
7	Ilheus	12.2 (8)	0.6 (3)	5.8	3.2		
8	None	148.3 (8)	72.0 (2)	30.0	34.1		
8	W. Nile	21.5 (10)	4.4 (3)	16.6	5.0		
8	Ilheus	25.7 (8)	5.9 (3)	13.2	14.6		
9	None	248.0 (3)	202.0 (2)	49.2	13.3	9.0	(8–12)
9	W. Nile	72.7 (4)	55.4 (2)	25.8	22.6	9.1	(8–10)
9	Ilheus	80.1 (6)	44.0 (3)	30.4	35.0	9.8	(9–10)
Virus Inoculated 6 Days after Ak4 Leukemia							
6	None	28.6 (10)	4.1 (3)	4.2	11.9		
6	W. Nile	27.1 (10)	8.7 (3)	3.6	6.1		
6	Ilheus	18.8 (10)	8.1 (3)	6.3	6.7		
7	None	90.7 (10)	43.3 (3)	13.2	27.1		
7	W. Nile	18.2 (6)	3.8 (3)	4.0	11.7		
7	Ilheus	49.2 (10)	11.8 (3)	7.1	14.1		
8	None	281.7 (7)	238.0 (3)	42.2	37.8		
8	W. Nile	25.3 (3)	6.5 (3)	2.8	16.1		
8	Ilheus	16.5 (9)	5.6 (3)	3.1	5.8		
9	None	341.0 (4)	201.0 (3)	38.8	23.5		
9	W. Nile	20.4 (2)	9.5 (2)	4.4	6.5		
9	Ilheus	42.3 (9)	20.6 (3)	3.1	8.8		
10	None	296.0 (3)	296.0 (3)	0	0		
10	W. Nile	98.2 (2)	66.5 (2)	10.0	21.5		
10	Ilheus	159.0 (5)	125.0 (3)	6.0	6.6		
12	None	325.0 (1)	325.0 (1)	0	0	9.7	(7–14)
12	W. Nile	(0)	(0)			8.2	(7–12)
12	Ilheus	268.0 (1)	268.0 (1)	0	0	10.7	(8–13)

* All blood counts are expressed as thousands of cells per cubic millimeter of blood. The figures in parentheses following the mean blood cell counts indicate the number of mice averaged, and in the leukocyte column this figure also indicates the numbers of mice surviving on that day. Since differential counts were not done on all mice, the sum of the average differential counts is not identical with the average total leukocyte counts.

† Survival times are computed from inoculation of leukemia to death, regardless of time of virus inoculation.

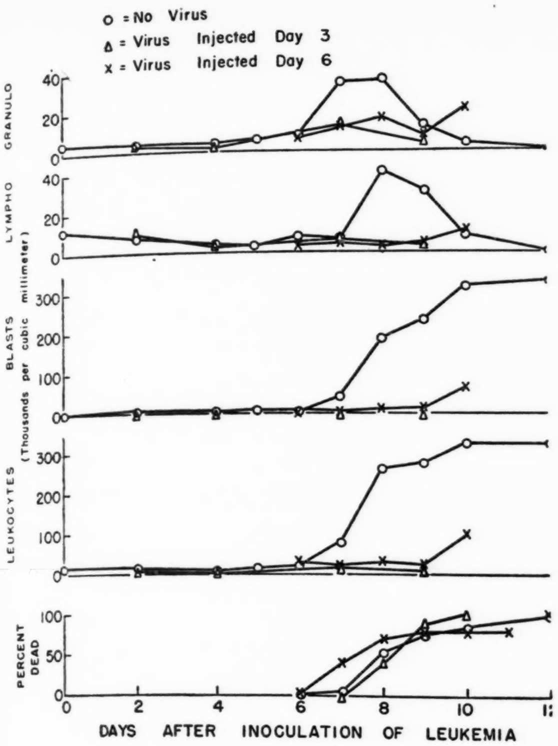


CHART 1.—The effects of West Nile virus infection on survival and blood picture of mice bearing Ak4 leukemia.

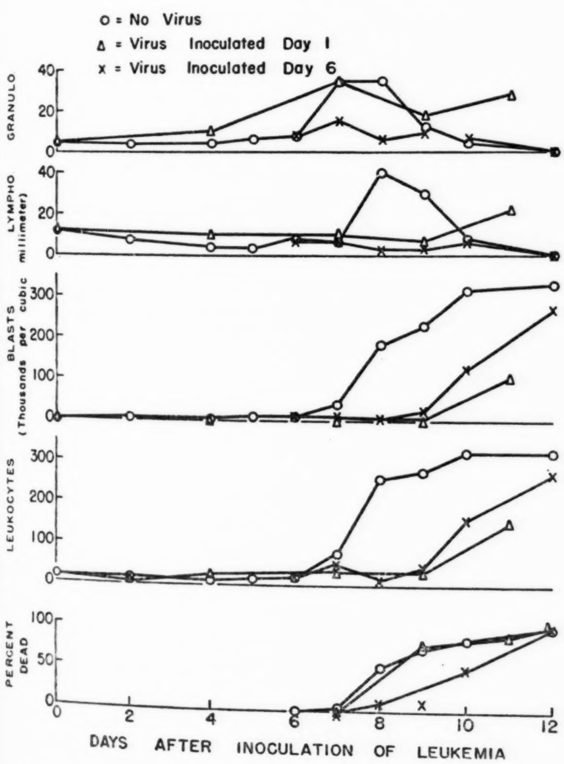


CHART 2.—The effects of Ilheus virus infection on survival and blood picture of mice bearing Ak4 leukemia.

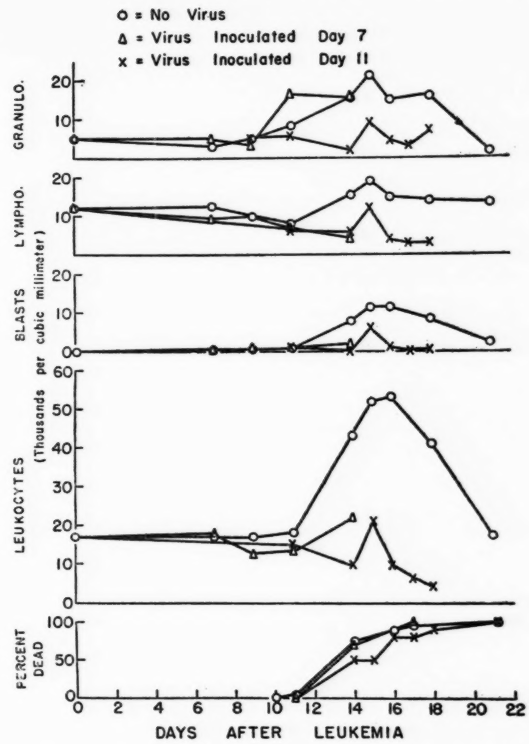


CHART 3.—The effects of West Nile virus infection on survival and blood picture of mice bearing Ak 9421 leukemia.

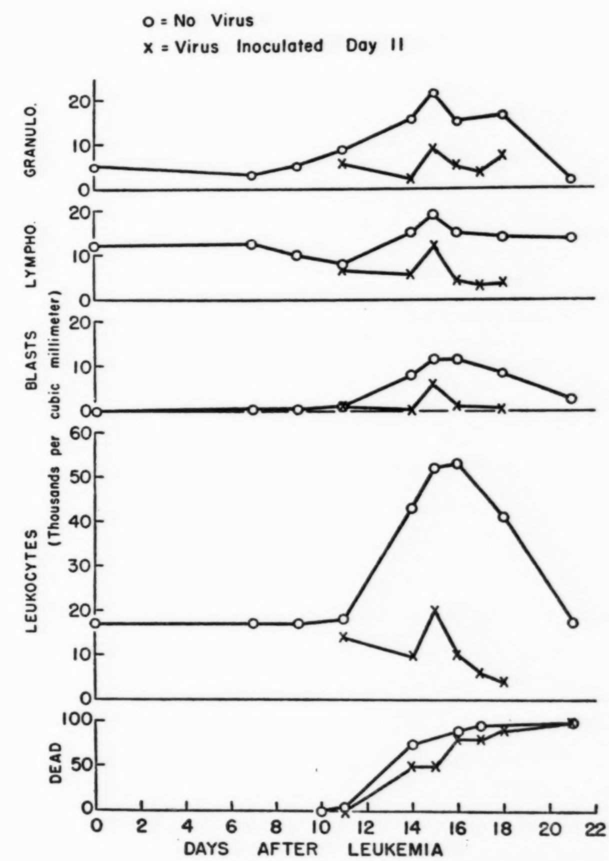


CHART 4.—The effects of Ilheus virus infection on survival and blood picture of mice bearing Ak 9421 leukemia.

studies with 9421 leukemia are not tabulated because of space limitations.) Inhibition of leukemic infiltration of liver and spleen was again apparent, since weights of these organs were within normal limits in the virus-infected mice at times when the control mice showed marked hepatosplenomegaly.

Effect of Ilheus virus on Ak 9421 leukemia.—When Ilheus virus was inoculated on the eleventh day of Ak 9421 leukemia, the leukemic leukocytosis was partially suppressed (Chart 4). When the virus was given on the seventh day, no effect on the course of the leukemia was observed.

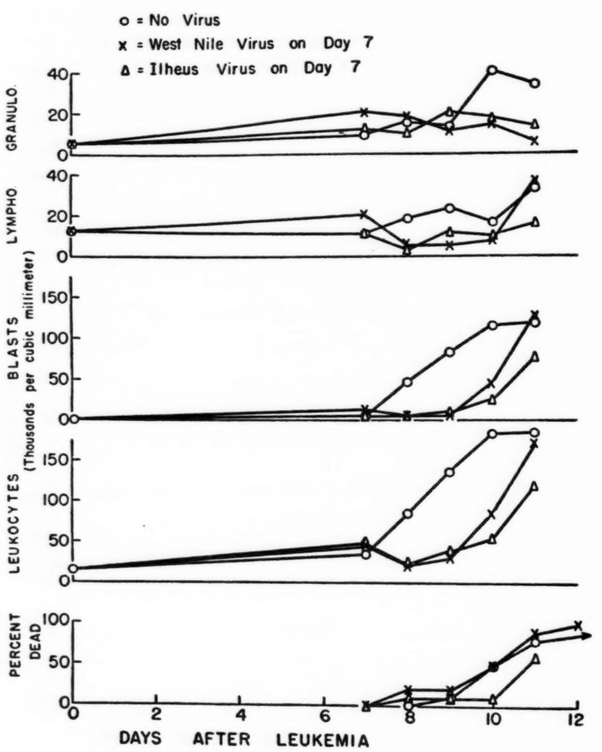


CHART 5.—The effects of West Nile and Ilheus virus infections on survival and blood picture of mice bearing Ak4R leukemia.

Effect of viruses on Ak4R leukemia.—Ak4R leukemia (3) differs from its parent strain Ak4 in that it is not affected by treatment with folic acid antagonists, whereas this treatment will cause a 100 per cent increase in survival time in the parent strain. This resistance to anti-folic compounds was produced by serial passage of Ak4 leukemia in A-methopterin–treated mice (2). In untreated mice the course of Ak4R leukemia is slightly longer than that of Ak4 leukemia, but in other respects it is almost identical.

West Nile virus or Ilheus virus inoculated on the seventh day after inoculation of Ak4R leukemia caused inhibition of leukemic leukocytosis for 3 days, as compared to untreated controls. Mice surviving beyond this period showed leukocytosis equal to that in the untreated controls. Survival time was not prolonged by either virus (Chart 5).

DISCUSSION

This work extends the spectrum of neoplasms which are inhibited by neurotropic viruses to include the mouse leukemias. This anti-neoplastic effect is evidenced by a delay of 2–3 days in the development of leukemic leukocytosis and visceral infiltration. The interval of 2–3 days represents 20–40 per cent of the total duration of this neoplastic process. Such an effect is analogous to the slowing of growth of localized tumors, such as the Sarcoma 180, which has previously been reported for these viruses (4). There was a very slight but quite consistent increase in survival time of the virus-treated mice in most of these studies. These differences are too small to have statistical significance in groups of only ten mice, but the occurrence of even a slight increase in survival is suggestive of inhibition of the leukemic process, when one remembers that the "treatment" is of itself rapidly lethal. It seems quite possible that other viruses might exhibit a greater anti-leukemic effect, and it may be that nonlethal viruses will have this effect, as has been reported for leukosis of fowl (9). It is clear from these data that these viruses exerted their anti-leukemic effect even when inoculated late in the course of the disease.

The mechanism of any anti-neoplastic effect is of utmost interest. The current excitement over the wide therapeutic effects of adrenal steroids makes every investigator alert to the possibility that his manipulations may cause adrenal hyperactivity. In this connection it is of interest that in studies now in progress with Semliki virus no anti-leukemic effect has been demonstrated. This virus is rapidly lethal in the mouse and hence might be expected to produce as great an adrenal stress as West Nile or Ilheus virus. Hence, it seems unlikely that the anti-leukemic effects observed with the latter viruses can be attributed to a nonspecific stress.

The observations indicating that the virus was in the leukemic cells (in spleen and blood) suggest that the viruses acted by entering and destroying, or preventing multiplication of, these cells. Even if we accept this as proved, however, the *mechanism* by which the virus inhibits the cells remains to be explained. The possibilities that the virus blocks an essential step in cellular metabolism or competes with the cells for essential metabolites deserve consideration. It is thought that the antineoplastic effects of various vitamin and purine

analogs is caused by such interference with metabolic processes. The fact that West Nile and Ilheus viruses were effective against both Ak4 and Ak4R leukemia indicates that the viruses do not act through the same mechanism as the folic acid antagonists.

SUMMARY

Infections with West Nile or Ilheus viruses were established in mice bearing Ak4, Ak4R, or Ak 9421 leukemia of varying duration. It was demonstrated that both of these viruses temporarily inhibit leukemic leukocytosis and infiltration, even when injected in the late stage of those leukemias. There was no significant increase in survival time in the virus-treated mice. Evidence was obtained which indicates that virus was present in, or on, the leukemic cells.

REFERENCES

1. BURCHENAL, J. H.; BIEDLER, J. L.; NUTTING, J.; and STOBBE, G. D. The Effects of 4-Amino-N[10]-methylpteroylglutamic Acid on the Leukocytes of the Normal and Leukemic Mouse. Blood, **5**:167–76, 1950.
2. BURCHENAL, J. H.; ROBINSON, E.; JOHNSTON, S. F.; and KUSHIDA, M. N. The Induction of Resistance to 4-Amino-N[10]-methyl-pteroylglutamic Acid in a Strain of Transmitted Mouse Leukemia. Science, **111**:116–17, 1950.
3. BURCHENAL, J. H.; WEBBER, L. F.; MEIGS, G. M.; and Biedler, J. L. A Comparison of the Effects of 4-Amino-N[10]-methyl-pterolyglutamic Acid and 2,6-Diaminopurine on Sensitive and Resistant Sublines of a Strain of Mouse Leukemia. Blood, **6**:337–43, 1951.
4. KAPROWSKI, H., and NORTON, T. W. Interference between Certain Neurotropic Viruses and Transplantable Mouse Tumors. Cancer, **3**:874–85, 1950.
5. LAEMMERT, H. W., and HUGHES, T. P. The Virus of Ilheus Encephalitis. Isolation, Serological Specificity and Transmission. J. Immunol., **55**:61–67, 1947.
6. MOORE, A. E. The Destructive Effects of the Virus of Russian Far East Encephalitis on the Transplantable Mouse Sarcoma 180. Cancer, **2**:525–34, 1949.
7. ———. Effect of the Inoculation of the Viruses of Influenza A and *Herpes simplex* on the Growth of Transplantable Tumors in Mice. *Ibid.*, pp. 516–24.
8. MOORE, A. E., and O'Connor, S. Further Studies on the Destructive Effect of the Virus of Russian Far East Encephalitis on the Transplantable Mouse Sarcoma 180. Cancer, **3**:886–90, 1950.
9. SHARPLESS, G. R.; DAVIES, M. C.; and COX, H. R. Antagonistic Action of Certain Neurotropic Viruses toward a Lymphoid Tumor in Chickens with Resulting Immunity. Proc. Soc. Exper. Biol. & Med., **73**:270–75, 1950.
10. SMITHBURN, K. C.; HUGHES, T. P.; BURKE, A. W.; and PAUL, J. H. A Neurotropic Virus Isolated from the Blood of a Native of Uganda. Am. J. Trop. Med., **20**:471–92, 1940.
11. TURNER, J. C., and MULLIKEN, B. Effects of Intravenous Vaccinia in Mice with Sarcoma 180 or Leukemia 9417. Cancer, **3**:354–60, 1950.

The Variable Expressivity of a Pigment Cell Gene from Zero Effect to Melanotic Tumor Induction*

MYRON GORDON

(*New York Zoölogical Society,*† *American Museum of Natural History, New York 24, N.Y.*)

Studies in human genetics have repeatedly shown the importance of the phenomena of penetrance and expressivity. It is said that a given gene has variable expressivity when the manifestations of a trait produced by that gene are different from individual to individual. The penetrance of a gene refers to the relative frequency with which a gene produces an effect. A dominant character, definitely known to be inherited, may not show up in a parent. That character finds expression in the members of subsequent generations when suitable genetic and environmental conditions are present. The problem of irregularities in the expression of genes in man is reviewed by Snyder (23) and Stern (24).

The purpose of this paper is to report the results obtained from experiments conducted to solve a somewhat similar problem involving animals of known and appropriate genetic constitutions. In the course of genetic study of normal and atypical pigment cell growth in fishes, platyfish were found that must have carried the sex-linked dominant gene (*Sd*) for macromelanophore-spotting of the dorsal fin, but the fish did not show any macromelanophores at all. The *Sd* platyfish were tested by mating them to swordtails, a related species that carries the recessive genes for the absence of macromelanophores. Swordtails, however, carry dominant *Sd* modifying genes which are capable of stimulating the growth of the macromelanophores in platyfish-swordtail hybrids. As a result of a series of matings which will be outlined, macromelanophores develop in the dorsal fins of some of the *Sd* platyfish-swordtail hybrids, while in those of other hybrids macromelanophores form melanomas at the same site.

* Aided by a grant from the National Cancer Institute, National Institutes of Health, Public Health Service for the project "Genetic and Correlated Studies of Normal and Atypical Pigment Cell Growth."

† From the Genetics Laboratory of the New York Aquarium, New York Zoölogical Society at the American Museum of Natural History, New York 24, New York.

Received for publication March 14, 1951.

THE GENES FOR MACROMELANOPHORES

The platyfish (*Platypoecilus maculatus*) has a series of five dominant, multiple alleles each of which controls the development of macromelanophores in a specific part of the body of the animal (Gordon [12]). Three of the five genes are involved in this experiment: (*a*) *Sd* for macromelanophores on the dorsal fin, (*b*) *Sp* for similar black pigment cells irregularly arranged on the sides of the body, and (*c*) *Sr* for macromelanophores arranged regularly in rows on the flanks.

All the macromelanophore patterns are present in the natural populations of the platyfish which are indigenous in the Atlantic coastal rivers of Mexico, Guatemala, and British Honduras. The various geographical populations of these fish are genetically distinct with regard to the distribution of their color pattern frequencies, according to the analysis of Gordon (11, 13). Their genetic distinctness has been confirmed by Gordon and Gordon (7), who calculated and compared the gene frequencies for seven micromelanophore color patterns. Recently, calculations were completed of five macromelanophore patterns in six natural populations of these fish. Again the distribution of the gene frequencies for these color patterns indicates that each population is genetically distinct. In this connection, an interesting comparison may be made with respect to man. According to the summary by Boyd (2), the races of man are separable on the basis of their gene frequencies for the inherited blood types.

The macromelanophore genes are sex-linked. Different natural populations of the same platyfish species may have opposing types of genetic mechanisms for sex determination. Members from the Rio Jamapa (Veracruz) population and those from the Rio Coatzacoalcos (Puerto Mexico) population have a genetic mechanism in which the female is homogametic, or XX, and the male is heterogametic, or XY. The mechanism therefore is somewhat like that found in man—all the more so, because in the platyfish and in man the Y chromosomes carry genes for pigment patterns.

In the platyfish from the Belize River of British Honduras, however, sex-linkage follows the pattern in which WY represents the female and YY the male. The latter system is somewhat like that found in birds in which the female is heterozygous and the male homozygous.

The distinctive feature of sex-linkage in fishes is that regardless of whether the system be XX–XY or WY–YY, all the sex chromosomes may carry dominant color genes, and crossing-over between homologous portions of the sex chromosomes occurs. However, the cross-over frequency is low, about 1 per cent, according to Gordon (10). It may be said, therefore, that the macromelanophore genes are incompletely, rather than completely, sex-linked. Again the parallelism between the type of sex-linked inheritance in man and fish is worthy of notice.

The following preliminary experiment establishes two points: first, that the XX–XY type of genetic sex determination is characteristic of fishes of the Rio Jamapa and Rio Coatzacoalcos populations; and, second, that a change occurs in the expressivity of the *Sd* gene for macromelanophore-spotting of the dorsal fin. The mating involved a stripe-sided female platyfish from the Rio Jamapa and a male spotted-dorsal platyfish of the same species (*Platypoecilus maculatus*) from the Rio Coatzacoalcos, as follows:

Experiment 1

Playfish female parent	Platyfish male parent
from Rio Jamapa	from Rio Coatzacoalcos (No. Cp 11)
(No. Jp 30^9-5)	
Striped-sided	Spotted-dorsal
(homozygous)	(heterozygous)[1]
(X)*Sr*/(X)*Sr*	(X)*Sd*/(Y)+

The spotting on the dorsal fin of the male parent was made up of a small group of macromelanophores that covered much less than one-half of the total area of the dorsal fin. Furthermore, the spots were mainly restricted to that fin; only a few were found on the body proper (Fig. 1).

The intensity of macromelanophore-spotting on the dorsal fins of the first generation offspring was strikingly different from that of their male parent. It will be noticed (Table 1) that the *Sd* trait appeared only in the female offspring, indicating the XX female–XY male type of sex-linkage. Also noteworthy is that the intensity of spotting in the daughters was always far greater than in their male parent. The term "spilling-over" in the table below refers to the degree to which the macromelanophores, by cell multiplication and migration, spread from the base of the dorsal fin into adjacent areas of the body proper (Fig. 2).

[1] The symbol "+", synonymous with *sd*, refers to the recessive state of the spotted-dorsal gene *Sd*.

Experiment 2 illustrates the influence of sex upon the expressivity of the spotted-dorsal trait. A male from the Rio Coatzacoalcos population, homozygous for the *Sd* gene, was mated to a female from the Rio Jamapa population, homozygous for the stripe-sided gene *Sr*. This mating may be expressed genetically as follows:

TABLE 1

THE AMOUNT OF MACROMELANOPHORE-SPOTTING ON THE DORSAL FINS IN THE FIRST GENERATION (Brood No. 270)

Intensity	Spread	Females (X)*Sr*/(X)*Sd*	Males (X)*Sr*/(Y)+
1.	None present	0	54
2.	"Spilling-over" slight	19	0
3.	"Spilling-over" intermediate	19	0
4.	"Spilling-over" intense	8	0
	Total	46	54

Experiment 2

Platyfish female parent	Platyfish male parent
from Rio Jamapa	from Rio Coatzacoalcos (No. Cp 30^2-11)
(No. Jp. 30^{10}-7)	
Stripe-sided	Spotted-dorsal
(X)*Sr*/(X)*Sr*	(X)*Sd*/(Y)*Sd*

All the F_1 fish had the stripe-sided trait (*Sr*), and most of them also had the spotted-dorsal feature (*Sd*)—that is, all but 13 out of a total of 150. Since 137 out of the 150 individuals expressed the pattern, the penetrance of the *Sd* gene may be said to be 91.3 per cent in this mating. The expressivity of the *Sd* character was extremely variable, ranging from zero, as represented by the thirteen fish just mentioned, to a stage far beyond normality—a stage in which the entire fin is blackened, and the macromelanophores "spill-over" from the dorsal fin to the ventral area of the body. Seven classes of pigmentation intensity were set up arbitrarily to estimate the variability of expression of the *Sd* trait. The frequency of each class and the sexes are indicated in Table 2 and Chart 1.

A male (*Platypoecilus maculatus*) from Brood No. 291 was selected, one out of the thirteen which had no visible macromelanophore on the dorsal fin. It was mated to a swordtail (*Xiphophorus hellerii*) from the Belize River stock. The chromosomes of the swordtail that are homologs of the

TABLE 2

THE AMOUNT OF MACROMELANOPHORE-SPOTTING ON THE DORSAL FIN IN BROOD NO. 291

Intensity	Spread	Females (X)*Sr*/(X)*Sd*	Males (X)*Sr*/(Y)*Sd*
1.	None	0	13
2.	One small spot on fin	0	21
3.	One-half of fin covered	0	20
4.	More than one-half	13	10
5.	"Spilling-over" slight	36	11
6.	"Spilling-over" intermediate	22	0
7.	"Spilling-over" intense	4	0
	Total	75	75

sex chromosomes of the platyfish are indicated as X′. The results were as follows:

Experiment 3

Swordtail female parent from Belize River (No. Bx 6)	Platyfish male parent from Rio Jamapa-Rio Coatzacoalcos mating (No. 291–11)
Unspotted	Unspotted dorsal fin, stripe-sided
(X′)+/(X′)+	(X)*Sr*/(Y)*Sd* (?)

The F_1 swordtail-platyfish hybrids fell into two pigmentary classes: first, those with macromelanophores only slightly intensified, arranged in rows along the flanks (*Sr*); and second, those with medium to intense macromelanophore pigmentation on the dorsal fin and on adjacent areas on the body (*Sd*). The hybrids are classified with reference to their pigmentary status in Table 3. In this table, the sexes of the animals are purposely omitted, owing to the fact that sex in many hybrids of this kind is indeterminate.

TABLE 3

MACROMELANOPHORE-SPOTTING IN BROOD NO. 303

Macromelanophores	Constitution	No.	Degree of pigmentation
Striped-sides	(X′)+/(X)*Sr*	14=14	Normal pigmentation
Spotted-dorsal fin	(X′)+/(Y)*Sd*	10=	3 with melanosis*
			7 with melanoma†

* *Melanosis* refers to a pathological state in which normal tissues are destroyed and replaced by macromelanophores; details in Reed and Gordon (22).

† *Melanoma* refers to a neoplastic state involving the abnormal growth of pigment cells in which the melanoblasts, as well as the macromelanophores, are the charasteristic cells. The abnormal growths are destructive and reach considerable size; details in Reed and Gordon (22), Gordon and Smith (16), and Levine (19).

Experiment 4

Using two fishes from the same stocks as used in Experiment 3, an unspotted swordtail (No. Bx 5) was mated to a platyfish (No. 291–12) having a tiny group of macromelanophores on the dorsal fin. They produced two groups of hybrids similar to the kind produced in Brood No. 303 (Table 4).

TABLE 4

MACROMELANOPHORE-SPOTTING IN BROOD NO. 302

Macromelanophores	Constitution	No.	Degree of pigmentation
Striped-sides	(X′)+/(X)*Sr*	6=6	Normal pigmentation
Spotted-dorsal fin	(X′)+/(Y)*Sd*	8=4	with melanosis
		4	with melanoma

Experiment 5

For control, a mating was made between a swordtail (No. Bx 1) and a platyfish (No. Bp Sd), both taken from the Belize River, British Honduras. The male platyfish had an intensely black dorsal fin, the most intensely black-spotted dorsal in the entire collection. The female swordtail's genetic constitution, written as before, was (X′)+/(X′)+, while the male platyfish, being homozygous for the Y chromosome, was (Y)+/(Y)*Sd*, as will be shown in Experiment 6. Their F_1 hybrids are classified in Table 5.

TABLE 5

MACROMELANOPHORE-SPOTTING IN BROOD NO. 301

Macromelanophores	Constitution	No.	Degree of pigmentation
None	(X′)+/(Y)+	19=19	Normal, gray
Spotted-dorsal fin	(X′)+/(Y)*Sd*	14=	4 with melanosis
			10 with melanoma

No appreciable differences existed in the pigmentation of the platyfish-swordtail hybrids from Experiments 3, 4, or 5. The differences in the expressivity of the *Sd* genes in the three original parental male platyfish were canceled out when the platyfish *Sd* genes were placed in association with the swordtails' *Sd* gene modifiers.

The following experiments again indicate clearly that specific genetic modifiers of the *Sd* gene exist in the platyfish as well as in the swordtail. A female platyfish of the Rio Jamapa population with the spot-sided gene *Sp* and spotted-dorsal gene *Sd* was mated to a male member of the Belize River population which was recessive for the macromelanophore genes. Macromelanophores were present on the sides and on the dorsal fin of the Rio Jamapa parent. This mating may be expressed as follows, and the results seen in Table 6.

Experiment 6

Platyfish female parent from Rio Jamapa (No. Jp 163²–6)	Platyfish male parent from Belize River (No. Bp 15)
Spot-sided, Spotted-dorsal fin	Recessive
(X)*Sp*/(X)*Sd*	(Y)+/(Y)+

One of the striking results obtained from the above experiment is that *all* the offspring are *males*. This can be explained by the assumption that the platyfish from the Belize River have a

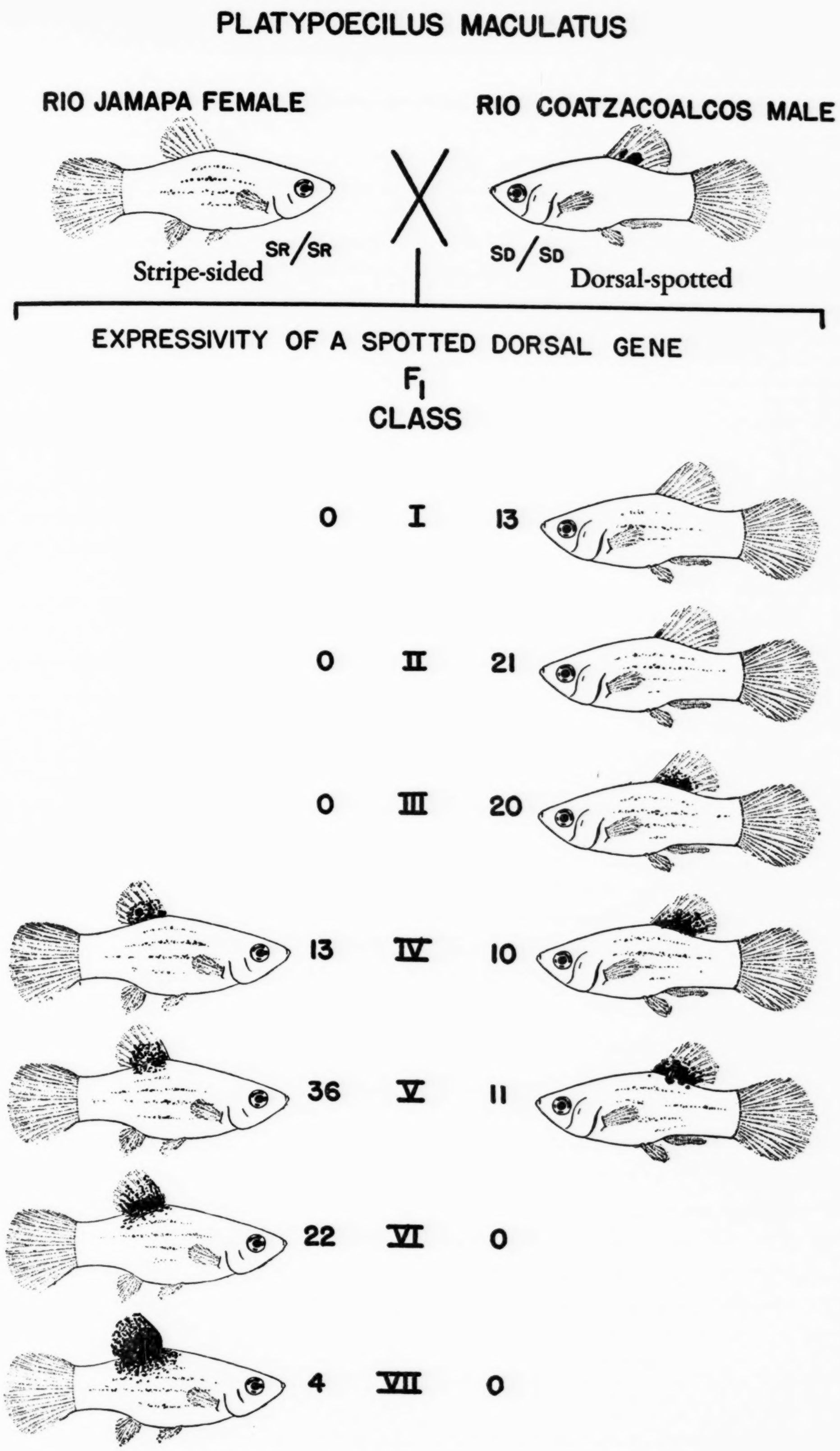


CHART 1.—The reduction in the penetrance of a macromelanophore gene, *Sd*, for spotted-dorsal fin and the increase in the *Sd* gene's expressivity in the hybrids obtained from a mating of two platyfishes, each from a geographically isolated natural population. Note that thirteen males fail to exhibit the spotted-dorsal pattern, although they carry the dominant gene for *Sd* for that pattern.

genetic sex-determining mechanism in which the female is WY and the male YY (Gordon, [14]). The situation in the platyfish of the Rio Jamapa is opposite, the female being XX and the male XY (Gordon, [10]). In Experiment 6, under consideration, an XX female was mated to a YY male. All their offspring are XY and therefore male. Somewhat similar results from other experiments in sex determination were described by Gordon (9).

TABLE 6

THE CONDITION OF MACROMELANOPHORE-SPOTTING IN BROOD NO. 305

	Females	Males	Theoretical genotypes
Faint spotting on sides	0	123	(X)*Sp*/(Y)+
Faint spotting on dorsal fin	0	2	(X)*Sd*/(Y)+
No spotting anywhere	0	114	(X)*Sd*/(Y)+ (?)
Total	0	239	

The second striking result was the reduction in the expressivity of the macromelanophore genes, the *Sp* gene being weakly represented, and the *Sd* gene being almost entirely suppressed. The penetrance of the *Sd* gene was low, being only 1.7 per cent. The calculation was made on the basis of the appearance of two visible spotted dorsal individuals, when 116 were expected.

If the number of *Sp* individuals in category 1 of Experiment 6 is compared with the total of those theoretically *Sd*, in categories 2 and 3, the expected 1:1 ratio of *Sp* to *Sd* animals is approached rather closely.

In order to check the true genotypes of fish in category 3, the next experiment was planned, designed to test for the presence of the *Sd* gene in the nonspotted but presumably (X)*Sd*/(Y)+ Rio Jamapa-Belize River hybrid platyfish. One of them with no visible macromelanophores was selected at random and mated to a wild swordtail. The mating may be expressed as follows:

Experiment 7

Swordtail female parent from Belize River	Platyfish male parent from Exp. 6 Rio Jamapa-Belize River hybrid
(No. Bx 2²-2)	(No. 305-12)
Unspotted	Unspotted
(X′)+/(X′)+	(X)*Sd*/(Y)+ (?)

Only four hybrids were obtained in the brood (No. 319). Two, presumably (X′)+/(Y)+, had no macromelanophores. The other two (X′)+/(X)*Sd* had definite spotted-dorsal fins at 2 weeks of age. When the *Sd* hybrids were 6 months old, the macromelanophores had grown so profusely that they "spilled-over" to the body proper. When they were 8 months old the dorsal fins were destroyed by the macromelanophores. Although the number of young obtained was extremely low, nevertheless the results indicate beyond doubt that the unspotted platyfish parent, taken at random and tested, indeed carried the *Sd* gene.

Thus, a parent with no visible sign of being a carrier of a potentially injurious dominant gene, transmitted that gene to its offspring. The potentially injurious gene, in association with modifiers received from its second parent, produced the definite pathological state leading to the formation of a melanoma.

Results from other experiments confirm, in part, the observations recorded above. A reduction in the expressivity of the *Sd* gene was found in the offspring of a mating between platyfish coming from two different laboratory stocks which was comparable to that observed in Experiment 6. Then the expressivity of apparently the same *Sd* gene was enhanced by means of mating the unspotted but presumably *Sd* platyfish to a swordtail.

The first part of the experiment concerned the following mating (see also Table 7).

Experiment 8

Platyfish female parent from Stock No. 239	Platyfish male parent from Stock No. 233
Recessive	Spotted-sides, Spotted-dorsal
(X)+/(X)+	(X)*Sp*/(Y)*Sd*

TABLE 7

MACROMELANOPHORE-SPOTTING IN BROOD NO. 262

	Theoretical genotypes: Females (X)*Sp*/(X)+	Males (X)+/(Y)*Sd*
Faint spotting on sides	9	0
Faint spotting on dorsal fin	0	1
No spotting anywhere	1	4
Total	10	5

Nine female offspring (X)+/(X)*Sp* had some macromelanophores on the sides of their bodies, but the number of pigment cells was far less than in their male parent, and one had none at all. Four of the five male offspring, presumably (X)+/(Y)*Sd*, showed no macromelanophores at all, although it was expected that all would have spotted dorsal fins. One of the five had a tiny black spot on the dorsal fin. The results obtained may be explained by assuming that the female platyfish from stock 239 carried a number of macromelanophore suppressor genes which affected the expressivity of the *Sp* and the *Sd* genes

of the male platyfish from stock 233. With regard to the *Sd* gene, the penetrance value is 20 per cent, and for the *Sp* gene it is 90 per cent, the penetrance value being calculated on the basis of minimum positive expressivity of the genes.

Two goals were set in Experiment 9. The first was to determine if the unspotted male intraspecific hybrids from Experiment 8 *did* have the genetic constitution (X)+/(Y)*Sd*. This could be checked by the reappearance of the spotted-dorsal character in their offspring. The second goal was to determine the influence of certain known autosomal genes upon the expressivity of the *Sd* gene in those offspring in which the spotted-dorsal reappeared. To this end, one of the stock 262 males, without any macromelanophore-spotting, but presumably (X)+/(Y)*Sd* was selected at random and mated to an albino swordtail (Chart 2).

The albino swordtail was of a special stock which was recessive for three color genes as follows:

1. *i*, for albinism when homozygous, produces a yellow-pink body coloration and pink eyes; homozygous gene *i* inhibits melanin formation (Gordon [12]).
2. *st*, for golden or lack of micromelanophores when homozygous, produces a yellow body color (Gordon [7]); *st*, in association with *i*, produces pink eyes; but in association with the dominant allele (of *i*) *I*, *st* produces black-eyed, golden-colored fishes. The dominant allele (of *st*) *St*, when *I* is present, produces black eyes and a gray body coloring. These combinations may be summarized as follows:

   i st = pink-eyed, pink body coloring
   i St = pink-eyed, pink body coloring
   I st = black-eyed, golden body coloring
   I St = black-eyed, gray body coloring
3. *sd*, for unspotted dorsal fins; this was previously written "+" rather than *sd;* both "+" and *sd* refer to the recessive state of the spotted-dorsal gene *Sd*.
4. In addition, the albino swordtail usually carries a number of macromelanophore stimulating genes, two of which are genetically indicated as *A* and *B*. Details concerning the action of these genes were presented by Gordon (12).

Thus, the mating between the albino swordtail and the unspotted platyfish, that was to be tested for the presence of the *Sd* gene, may be expressed genetically as follows (the symbols for the sex chromosomes are purposely excluded):

Experiment 9

Albino Swordtail female (182^2–3)	Platyfish male (262–11)
Color: pink-eyed, pink body	Color: black-eyed, gray body
sd i st A B / sd i st A B	*Sd I St a b / sd I St a b*

TABLE 8

THE CONDITION OF PIGMENTATION IN INTERSPECIFIC HYBRIDS, BROOD NO. 285

No.	Genotypes	Color
8	*sd i st A B / sd I St a b*	=Black-eyed, gray-bodied with unspotted dorsal fins
14	*sd i st A B / Sd I St a b*	=Black-eyed, gray-bodied with *spotted-dorsal* fins

The fact that fourteen of the first generation platyfish-swordtail hybrids had macromelanophores in the dorsal fin indicates that the dominant gene *Sd* had not been wanting in their unspotted male platyfish parent. The results suggest that in the platyfish parent, the *Sd* gene's penetrance, through interaction with its suppressor modifiers, had been reduced radically, and as a consequence the expressivity of *Sd* gene was zero.

In the platyfish-swordtail hybrids, on the other hand, the penetrance of the *Sd* gene was elevated sharply, presumably by the action of the macromelanophore gene modifiers *A* and *B*. As a result, the spotted-dorsal trait reappeared.

One of the newly obtained spotted-dorsal (*Sd*) platyfish-swordtail hybrids was then backcrossed to an albino swordtail to determine whether the level of expressivity could be stepped up further in the backcross hybrids. The second mating in this series may be expressed as follows:

Experiment 10

Albino Swordtail female (182^4–9)	Platyfish-Swordtail hybrid (285–11)
sd i st A B / sd i st A B	*sd i st A B / Sd I St a b*

The twenty-nine backcross hybrids constituting Brood No. 311 were classified into six phenotypic groups, as follows:

1a. *sd i st*
1b. *sd i St* } = 3, Albinos, pink-eyed
2a. *Sd i st*
2b. *Sd i St* } = 3, Albinos, pink-eyed (dorsal fins red in color [see below])
3. *sd I st* = 4, Golden, black-eyed
4. *sd I St* = 5, Gray, black-eyed
5. *Sd I st* = 6, Golden, black-eyed, black spotted-dorsal fins variously affected:
   4, Spotted, normal dorsal fin
   2, Slight melanosis in dorsal fin

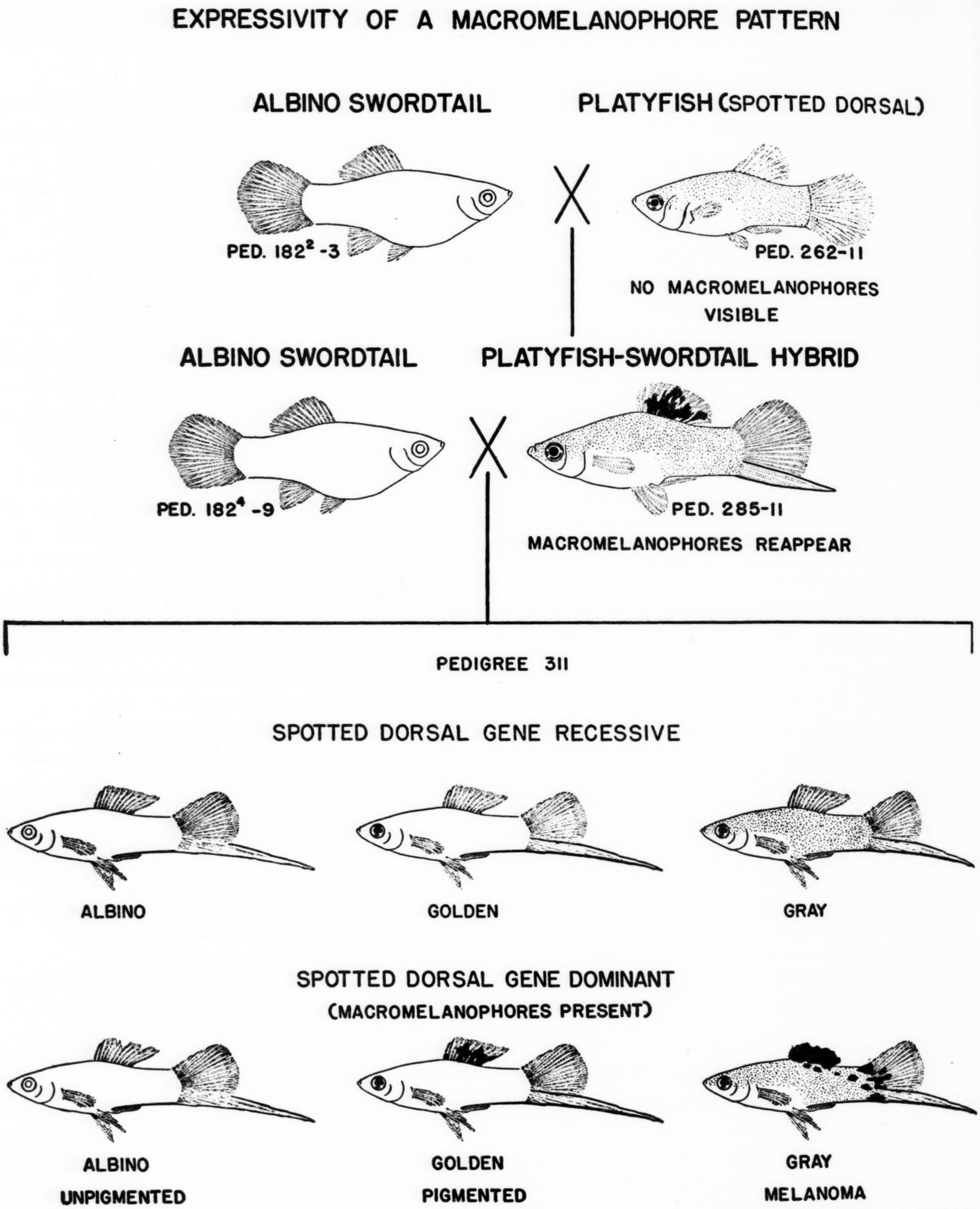


CHART 2.—The variable expressivity of the pigment cell gene *Sd* from zero effect to melanotic tumor induction. Note that the platyfish on the top line has no visible macromelanophores in its dorsal fin or elsewhere; its platyfish-swordtail hybrid offspring has the spotted-dorsal pattern; one of its backcross hybrid offspring, at the lowermost right-hand corner, has a melanoma of the dorsal fin.

6. *Sd I St* = 8, Gray, black-eyed, black spotted-dorsal fins variously affected:
   - 4, Slight melanosis in dorsal fin
   - 2, Severe melanosis in dorsal fin
   - 2, Melanoma in dorsal fin

With regard to the presence and degree of macromelanophore-spotting in the dorsal fin, the entire range of expressivity from zero to melanoma development is found in the seventeen backcross hybrids which theoretically carry the *Sd* gene. The significant point is that some of them have severe melanosis, and some of them are melanomatous.

The results presented above may be evaluated, first, for the influence of genes *A* and *B* on the expressivity of the *Sd* gene and, second, for the specific influence of other known genes, particularly the golden gene *st* and the albino gene *i* on *Sd*'s expressivity.

1. Phenotypes in group 1 (*a* and *b*) are albinos, owing to the presence of the homozygous *i* gene. Since the spotted-dorsal gene is recessive, *sd*, no manifestation of this gene is expected.

2. Phenotypes in group 2 (*a* and *b*) are also albinos for the same reason; they are homozygous for the *i* gene. Groups 1 and 2 are visibly different, however, owing to the fact that another sex-linked gene closely linked to *Sd*, *Dr* for red dorsal fin, previously not indicated, was also used in this experiment. The homozygous *i* gene does not inhibit the development of xantho-erythrophores which are responsible for the *Dr* red coloring, because these pigment cells contain color elements (pterins) which are chemically quite different from the melanins, according to Goodrich, Hill, and Arrick (4). Thus, all the albinos in group 2 had red dorsal fins and, because of the linkage of *Dr* and *Sd*, one might expect that they should also carry *Sd* gene. One of the *DrSd* hybrids showed a slight but significant abnormality in the dorsal fin (Fig. 3). This was probably brought about by the interaction of the *Sd* gene and its modifiers. With respect to melanin formation in their albino hybrids, the gene *i* is quite as effective as the *Sd* gene suppressors mentioned in Experiment 6.

3. Phenotypes in group 3 have black eyes and normal dorsal fins; the body color is yellow, owing to the homozygous *st* gene which prevents the full development of melanin in micromelanophores. Spotted-dorsal fishes are not expected here, because the *sd* gene is recessive.

4. Phenotypes in group 4 have the plain, wild color pattern. They have black eyes, micromelanophores in their skin which produce gray body color. Their dorsal fins are unspotted, because the *sd* gene is recessive.

5. Phenotypes in group 5 have golden body color, but their dorsal fins contain macromelanophores (*Sd*). In some, these large black pigment cells have created an abnormal condition or melanosis, in which the melanophores, through their growth process, destroy and replace normal cells.

6. Phenotypes in group 6 contain two individuals out of eight which have melanomas in their dorsal fins (Fig. 3). This pathological condition has been induced genetically by the interaction of at least five genes:
   a. *Sd*, for macromelanophores in the dorsal fin
   b. *I*, for melanin production
   c. *St*, for melanin production in micromelanophores
   d. *A*, for one macromelanophore gene modifier
   e. *B*, for the second macromelanophore gene modifier

The complex of four genes, *I*, *St*, *A*, and *B*, apparently has overcome whatever *Sd*-gene-suppressors that the *Sd* platyfish parent number 262–11 had originally.

Owing to the backcrossing of the platyfish-swordtail hybrid (285–11) to the swordtail, their progeny under the pedigree 311 must have carried a high frequency of the swordtail macromelanophore gene modifiers *A* and *B*. This was tested by mating one of the backcross platyfish-swordtail hybrids of pedigree 311 to an albino swordtail. Hybrid male number 311–11, with a melanoma of its dorsal fin, and the albino swordtail produced, in Experiment 11, the following numbers and kinds of offspring which carry the pedigree number 334:

1a. *sd i st*
1b. *sd i St* } = 2, Albinos, pink-eyed

2a. *Sd i st*
2b. *Sd i St* } = 0, Albino, pink-eyed, red dorsal fins

3. *sd I st* = 1, Golden, black-eyed
4. *sd I St* = 4, Gray, black-eyed
5. *Sd I st* = 2, Golden, black-eyed, black dorsal fin
   - 1, Severe melanosis, dorsal fin partially destroyed
   - 1, Melanoma of dorsal fin
6. *Sd I St* = 3, Gray, black-eyed, black dorsal fin
   - 1, Severe melanosis, dorsal fin partially destroyed
   - 2, Melanoma of dorsal fin

As expected, the number and kinds of offspring of pedigree 334, for the most part, parallel the number and kinds that make up pedigree 311. The significant point is that the frequency of melanomas and severe melanosis is higher in the hybrids

of 334. This is probably the result of the accumulation of *Sd* modifiers *A* and *B*. It is likely that the hybrid 311–11 was homozygous for *A* or *B*, or both *A* and *B*.

It is most unlikely that this type of mating following the backcross system has any parallel in human marriage patterns. Yet in small communities, as in any small population with a measure of inbreeding, there would be a trend toward the accumulation of those genes in new generations that are found most frequently in the parents. In other words, the effects of genetic drift would be noticed.

DISCUSSION

The results of these experiments and those which have preceded them may be explained within the framework of the principles of Mendelian inheritance. The dorsal fin melanomas may be referred to the specific macromelanophore gene *Sd* in combination with a series of *Sd* modifiers, *A* and *B*. Undoubtedly many polygenic modifiers are involved, but they have not as yet been identified.

The variable effects of genes may be indicated in Timoféeff-Ressovsky's (25) terms of the conditions of expressivity and penetrance. The penetrance of a gene refers to the frequency with which the gene for a particular effect shows any phase of that effect; it is usually stated in terms of per cent. With reference to the spotted-dorsal gene, Gordon (9) has shown that in nine experiments in which the action of the *Sd* gene from the Rio Jamapa platyfish could be traced, the *Sd* gene has 100 per cent penetrance. Penetrance for the Rio Jamapa platyfish gene *Sd* falls to 91.3 per cent in the Rio Jamapa-Rio Coatzacoalcos hybrid platyfish (Experiment 2), and falls to only 1 per cent in the Rio Jamapa-Belize River hybrid platyfish (Experiment 6). The penetrance of the *Sd* gene seems to be influenced by its internal environment, as well as by its specific gene modifiers. For example, while the value of penetrance for the Rio Jamapa platyfish *Sd* gene is 100 per cent in the females of the Rio Jamapa-Rio Coatzacoalcos hybrid platyfish, it is only about 83 per cent in the males (Experiment 2). At the same time, as the value of *Sd*'s penetrance falls in the brood as a whole, the range of the gene's expressivity increases.

Since the degree of penetrance of the *Sd* gene in platyfish is usually calculated on the basis of the presence or absence of the appropriate pattern, it may be assumed that if the pattern is wanting, the cells that form that pattern are also wanting. The situation is quite different in albino platyfish-swordtail hybrids. The homozygous albino gene *i* is capable of inhibiting melanin formation, but it cannot prevent the growth of the macromelanophores. For example, Gordon (12) indicated that in albino platyfish-swordtail hybrids carrying *Sd* as well as homozygous *i* genes, the development of melanomas of the dorsal fin is not prevented. The melanomas of albinos are not typically black but are amelanotic. In this respect they resemble the amelanotic melanomas of man and mouse.

A somewhat similar reduction of the effect of a sex-linked gene for a melanin pattern by an autosomal gene in *Lebistes* was described by Goodrich, Hine, and Lesher (5) and by Goodrich, Josephson, Trinkaus, and Slate (6).

Levine (19) and Gordon (12) have pointed out that the *Sd* melanomas that occasionally develop on or near the caudal peduncle and fin show histological details of greater malignancy than the original ones on the dorsal fin. This reflects the importance of the environmental or regional influence in the invasive and destructive ability of a genetic type of melanotic tumor. Malignancy is manifested first by invasive growths and then by the destructive action of the pigment cells directly upon adjacent fascial, muscle, blood vessel, and periosteal cells. In the wake of these degenerative changes, which are often accompanied by hemorrhage and cachexia in melanomatous fishes, death follows.

Macromelanophores in their growth process in the *Sd I St A B* hybrids provide no additional evidence of the cellular "infectivity" of colorless dendritic cells by pigmented ones as described by Billingham and Medawar (1) in the guinea pig.

The severity of atypical pigment cell growth in hybrids between members of two geographically distinct platyfish populations is not as great as in the interspecific hybrid fishes. Gordon (15) suggested that this may be because the polygene modifiers of macromelanophore growth in members of isolated populations of the same species are not as divergent in their effects as those that occur in the members of distinct species.

Hybridization studies in mice indicate a somewhat comparable situation. Cloudman and Little (3) indicated that sarcoma of the uterus was not found either in members of the DBA or the short-tailed strains of *Mus musculus*. When mated, sarcoma of the uterus was found in a significant number of the first generation hybrids. Later, Little (20) showed that when a member of *Mus bactrianus*, a small, slowly maturing, relatively infertile species with no record of nonepithelial tumors, is mated with a member of the JAX C57 black *Mus musculus*, a large, rapidly maturing, fertile species susceptible to tumors, the incidence for both epi-

thelial and nonepithelial tumors in their first generation hybrids increased. Heston (17, 18) in a discussion of parallels in fish and mouse cancer genetics revealed that in his experimental colony ten mice with tumors of the Harderian gland were found and all ten were hybrids from a specific cross between members of the C3H and C57 black strains.

In referring to the work on the increased incidence of tumors in hybrid mice, Little (21) suggested that the control of growth in the hybrids is far less complete than in either parent strain. One of the major causes of unbalance in the internal environment of hybrids which leads to disease may be attributed to the conflict between different genetic trends or degrees of function. It is the task of geneticists to resolve the nature of these genetic factors. The relationship of these laboratory studies to human biology was stated by Little, who observed that in the United States, which contains an extraordinary mixture of biological types, and in which various degrees of crossing of dissimilar human populations is proceeding and will continue on a large scale, the biological study of hybrid animals has special importance. It is in this important field of population genetics that the use of unmixed, natural geographical populations of the platyfish may contribute to our further knowledge of the effects produced by the interaction of genes and their polygenic modifiers which originate independently in diverse geographical groups.

SUMMARY

The spotted-dorsal pattern is composed of a number of macromelanophores in the dorsal fin of the common platyfish, *Platypoecilus maculatus*. The spotted-dorsal pattern may be referred to the *Sd* gene which is dominant and sex-linked (or, more accurately, incompletely sex-linked). *Sd* belongs in an allelic series of four other somewhat similar genes, each of which controls the development of macromelanophores somewhere on the body of the platyfish.

The spotted-dorsal pattern is found in each of the six known populations of the platyfish in six large rivers of southern Mexico, Guatemala, and British Honduras. In tests, the penetrance of the spotted-dorsal gene in members of its own population is complete. The pattern varies in its expressivity; in some individuals it may be recognized by the presence of a tiny black spot in the dorsal fin which is produced by two or three macromelanophores; in others most of the fin is blackened by many large pigment cells. There are all intermediate degrees of expressivity between these extremes.

The spotted-dorsal gene shows a lower penetrance and greater range of expressivity in the first generation hybrids between members of two natural populations of platyfish. Some hybrids that carry the dominant *Sd* gene do not show the pattern at all. When an *Sd* platyfish with no pattern is mated to a swordtail, *Xiphophorus hellerii*, their *Sd* interspecific hybrids show the pattern in somewhat exaggerated form. When an *Sd* platyfish-swordtail hybrid is backcrossed to a swordtail, some of the *Sd* hybrids of the backcross generation develop melanotic tumors of the dorsal fin.

The genetic analysis of the process by which a dominant gene for macromelanophore production shifts from no macromelanophore formation to the development of melanomas shows that several types of modifying genes are involved. The members of the various natural populations of the platyfish apparently have many polygene modifiers, some of which increase and others decrease *Sd*'s expressivity. These effects are small compared to the modifying effects of genes of the swordtail.

In addition to the modifying genes which have no other detectable effects of their own, the macromelanophore genes are influenced by color genes like the albino gene and the golden gene.

The hereditary control of melanomas in hybrid fishes rests not on one gene but on a constellation of genes. To be effective, this assemblage of genes must contain a macromelanophore gene.

ACKNOWLEDGMENTS

The author thanks Olga Aronowitz, James W. Atz, T. R. Marcus, and D. E. Rosen of this laboratory for reading the manuscript, S. C. Dunton for the photographs, and the American Museum of Natural History for laboratory facilities.

REFERENCES

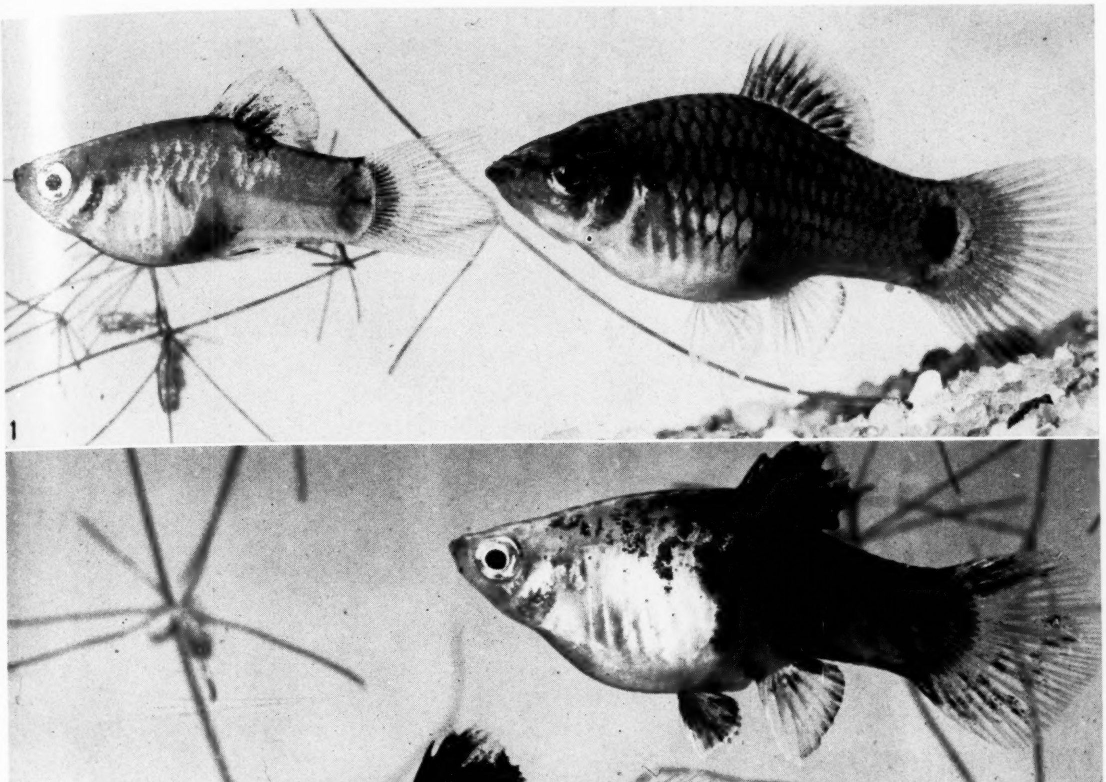
1. BILLINGHAM, R. E., and MEDAWAR, P. B. "Infective" Transformations of Cells. Brit. J. Cancer, **2**:126–31, 1948.
2. BOYD, W. C. Genetics and the Races of Man, pp. 1–453. Boston: Little, Brown & Co., 1950.
3. CLOUDMAN, A. M., and LITTLE, C. C. The Genetics of Tumour Formation in Mice in Relation to the Gene T for Brachyury. J. Genetics, **32**:487–504, 1936.
4. GOODRICH, H. B.; HILL, G. A.; and ARRICK, M. S. Chemical Identification of Gene-Control Pigments in *Platypoecilus* and *Xiphophorus* and Comparisons with Other Tropical Fish. Genetics, **26**:573–86, 1941.
5. GOODRICH, H. B.; HINE, R. L.; and LESHER, H. M. The Interaction of Genes in *Lebistes reticulatus*. Genetics, **32**:535–40, 1947.
6. GOODRICH, H. B.; JOSEPHSON, N. D.; TRINKAUS, J. P.; and SLATE, J. M. The Cellular Expression and Genetics of Two New Genes in *Lebistes reticulatus*. Genetics, **29**:584–92, 1944.

7. Gordon, H., and Gordon, M. Colour Patterns and Gene Frequencies in Natural Populations of a Platyfish. Heredity, **4**:61–73, 1950.
8. Gordon, M. Pigment Inheritance in the Mexican Killifish. Interaction of Factors in *Platypoecilus maculatus*. J. Hered., **19**:551–56, 1928.
9. ———. Interchanging Genetic mechanisms for Sex Determination in Fishes under Domestication. *Ibid.*, **37**:307–20, 1946.
10. ———. Genetics of *Platypoecilus maculatus*. IV. The Sex Determining Mechanism in Two Wild Populations of the Mexican Platyfish. Genetics, **32**:8–17, 1947.
11. ———. Speciation in Fishes. Distribution in Time and Space of Seven Dominant Multiple Alleles in *Platypoecilus maculatus*. In Adv. in Genetics, **1**:95–132, 1947.
12. ———. Effects of Five Primary Genes on the Site of Melanomas in Fishes and the Influence of Two Color Genes on Their Pigmentation. In The Biology of Melanomas, Spec. Publ. N.Y. Acad. Sc., **4**:216–68, 1948.
13. ———. Reunion after 300,000 Years. Animal Kingdom, **52**: 118–25, 1949.
14. ———. Genetics and Speciation in Fishes. Am. Phil. Soc. Year Book, pp. 158–59, 1949.
15. ———. The Origin of Modifying Genes That Influence the Normal and Atypical Growth of Pigment Cells in Fishes. Zoologica, **35**:19–20, 1950.
16. Gordon, M., and Smith, G. M. Progressive Growth Stages of the Heritable Melanotic Neoplastic Disease in Fishes from the Day of Birth. Am. J. Cancer, **34**:255–72, 1938.
17. Heston, W. E. Genetics of Cancer. In Adv. in Genetics, **2**:99–126, 1948.
18. ———. In Proceedings of the Second Conference on the Biology of Normal and Atypical Pigment Cell Growth. Zoologica, **35**:20, 1950.
19. Levine, M. The Cytology of the Typical and Amelanotic Melanoma. In The Biology of Melanomas, Spec. Publ. N.Y. Acad. Sc., **4**:177–215, 1948.
20. Little, C. C. Hybridization and Tumor Formation in Mice. Proc. Nat. Acad. Sc., **25**:452–55, 1939.
21. ———. Parental Influence. In Genetics, Medicine and Man. Ithaca: Cornell Univ. Press, 1947.
22. Reed, H. D., and Gordon, M. The Morphology of Melanotic Over-growths in Hybrids of Mexican Killifishes. Am. J. Cancer, **15**:1524–46, 1931.
23. Snyder, L. H. Medical Genetics, pp. 1–130. Durham: Duke Univ., 1941.
24. Stern, Curt. Principles of Human Genetics, pp. 1-617. San Francisco: W. H. Freeman & Co., 1949.
25. Timoféeff-Ressovsky, N. W. Gerichtetes Variieren in der phänotypischen Manifestierung einiger Genovariationen von *Drosophila funebris*. Naturw. Wchnschr., **19**:493–97, 1931.

Fig. 1.—A male and female platyfish (*Platypoecilus maculatus*). The male, to the left, originated in a stock obtained from the Rio Coatzacoalcos. Note the spotted-dorsal pattern, *Sd*. The female, to the right, originated in a stock from the Rio Jamapa. The female has the striped-sided pattern, *Sr*. This pair produced brood of the pedigree 270.

Fig. 2.—The third-generation platyfish offspring of the original pair shown in Figure 1. Note the extreme extension of the spotted-dorsal pattern. Some degree of melanosis in these intraspecific hybrids is evident, for part of the dorsal fin has been destroyed.

Fig. 3.—Two platyfish-swordtail hybrids from brood No. 311. On the left, an albino hybrid with the spotted-dorsal gene. Note that a small part of its dorsal fin has been destroyed even though no black pigment cells are visible. On the right, a black-eyed platyfish-swordtail hybrid with a typical melanoma of the dorsal fin.


1
2
3

Relation of Mitotic Activity to the Effects of X-Rays and Nitrogen Mustard as Indicated by the Growth of Corn Seedlings*

MARY A. RUSSELL AND FRANCIS J. MICHELINI†

(Department of Biological Sciences, University of Delaware, Newark, Del.)

In their review of the early research on mustard compounds, Gilman and Philips (3) list several striking similarities between the biological effects of these chemicals and radiation. They report that "Actively proliferating cells are selectively vulnerable to the cytotoxic action of the mustards." There is general agreement that, in most cases, dividing cells are more sensitive to radiation than resting cells, but recent literature indicates a conflict of opinion as to whether dividing cells are necessarily more sensitive to the effects of mustards. Friedenwald and Buschke (2) have discussed the difficulty of the problem. They point out that while some tissues with many dividing cells, such as bone marrow and intestinal mucosa, are very sensitive to mustard, a tissue like liver, with active cell division stimulated by partial hepatectomy, is comparatively insensitive.

The purpose of this investigation is to compare the sensitivity of tissue containing many dividing cells with the *same kind of tissue* in which little or no cell division is present at the time of exposure to either x-rays or nitrogen mustard. The root of the corn seedling has been found to be a satisfactory organism for studying the effects of both x-rays (5) and nitrogen mustard (6). It is particularly suitable for this problem, because tissue may be obtained with no cell division or with varying percentages of dividing cells by controlling the length of the germination period or by chemical treatment.

MATERIALS AND METHODS

Corn seed of the "U.S. 13" strain was treated for 5 minutes in a 0.05 per cent mercurophen solution to prevent the growth of mold. After soaking in water for 1 hour, the seed was allowed to germinate at 30° C. Tall 600-ml. beakers were lined with filter paper, then loosely filled with moist sphagnum moss (buffered to maintain a pH of about 5), covered with petri dish lids, and autoclaved at 15 lb. pressure for 30 minutes. After the desired period of germination, the seedlings were exposed to x-rays or to a solution of methylbis(β-chloroethyl)amine hydrochloride, designated as HN2. After treatment, about fifteen seedlings were placed in each beaker so that the roots grew down between the paper and the glass. The beakers were incubated at 30° C. A mark was placed on every beaker to indicate the position of the first seedling, so that a record could be kept of each individual. The length of the primary roots was generally measured twice daily, and observations were made at more frequent intervals to determine the time at which the first lateral roots appeared. If the primary roots reached the bottom of the beaker (about 120 mm.), they were classed as survivors, and roots whose length remained constant for 3 days were recorded as having stopped growing.

The x-ray machine used in these experiments was operated at 120 kv. and 10 ma with a 1.5-mm. aluminum filter. The output intensity was 72 r/min. During radiation the seeds were placed with embryo side up and covered with cellophane to prevent dehydration.

The HN2 used in these investigations was furnished by the Army Chemical Center, Maryland. A fresh solution was prepared just before each experiment, with distilled water of about pH 5.7, unless otherwise stated. A temperature of 28°–30° C. was maintained during exposure of the seedlings to HN2, and they were washed for 5 minutes in running tap water to remove all traces of the chemical.

Two methods were used to obtain tissue with little or no cell division for comparison with similar tissue containing a higher percentage of dividing cells.

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Delaware (NR 164-858).

† Present address: Botany Department, University of Pennsylvania, Philadelphia, Pa.

Received for publication March 16, 1951.

Method A

Use of embryos germinated for different periods of time.—Stained sections of the radicle tips of embryos germinated for 12 hours showed no mitotic figures, but after 15 or 16 hours of germination, about 15 per cent of the cells in the meristematic region of the tip were in some stage of mitosis. Therefore, 15 or 16 hours were allowed for germination when tissue with cell division was desired, and 11 or 12 hours when the experiment called for tissue without cell division. These two germination periods were chosen because it was desirable to use tissue in as nearly the same physiological condition as possible, but at the same time to make certain that the 4 hours' difference would divide the two groups into one with cell division and one without cell division. In preparing for these experiments, one set of seeds was started germinating 4 hours before the other, so that both sets would be ready for exposure to either x-rays or HN2 at the same time. In the HN2 experiments, controls were placed in distilled water during the time when the seedlings were soaked in the HN2 solution.

Method B

Use of seedlings treated with 3,5-dinitro-o-cresol (DNC), to inhibit cell division for comparison with normal seedlings.—Clowes and Krahl (1) reported that DNC was a reversible mitotic inhibitor when its action was tested on sea urchin eggs. Beck and Russell[1] later found that corn roots would resume normal growth after cell division was decreased in the root tip by means of exposure to DNC. Seedlings with roots about 10 mm. long were divided into two groups, as follows: the roots of group 1 were immersed in a 10^{-5} M solution of DNC for 19 hours at 20° C. and washed in running water for 5 minutes; the roots in group 2 were kept in distilled water for the same time at the same temperature. Both groups were then ready for exposure to either radiation or HN2. During the soaking period, the DNC-treated roots showed no appreciable growth, whereas the controls grew normally. Charts 2 and 3 show that the DNC-treated roots quickly recovered, and their growth soon paralleled that of the controls. It was noted in sections prepared from root tips fixed at the end of the 19-hour soaking period that about 22 per cent of the total cells in the meristematic region were dividing in group 1, while in the normal group (2) about 38 per cent were in some stage of division. Part of each group was kept untreated as controls.

[1] L. V. Beck and M. A. Russell, unpublished report, 1934.

RESULTS

Table 1 shows that, in every experiment, the percentage of roots which stopped growing was always higher in the group of seedlings with cell division present at the time of radiation. When the averages from all the experiments are compared, the difference is very striking ($P < 0.001$). A study of the lateral roots showed that the seedlings treated at a time after cell division had started were more sensitive to radiation than the seedlings in which there was no cell division at the time of treatment. In the group in which cell division was present, only 60 per cent of the seedlings produced laterals, while in the second group 97 per cent had laterals. In the first group, the initial appearance of the laterals was delayed longer than in the second group. All these facts indicate that the seedlings in the group with cell division suffered the greater amount of radiation injury.

In Table 2, the behavior of the primary roots after treatment with HN2 shows that there was much less difference in sensitivity to this agent between the group with dividing cells and the group with no cell division at the time of exposure to the HN2 solution. The difference is not significant ($0.01 < P < 0.02$). A study of the lateral roots showed that, although the first of these appeared in both groups at the same time, they were more numerous in the group without cell division, showing these seedlings to be slightly more resistant to HN2.

Chart 1 shows the growth curves of the primary roots which died in two typical experiments using Method A (Table 1, Exp. 3, and Table 2, Exp. 3). The shape of the curve made from data from roots treated with HN2 is very similar to the curve made from the data from the x-rayed roots, and the final length of the primary roots is about the same in both cases.

Chart 2 shows the behavior of the primary roots in a typical experiment (Exp. 3) from Table 3. In each experiment of this series, a larger percentage of roots stopped growing in the normal cell division group than in the reduced cell division group. The difference is highly significant ($P < 0.001$). Again, the laterals appeared later in the former group, and at the end of the experiment only 83 per cent of these seedlings had laterals, as compared to the presence of laterals in 99 per cent of the group with reduced mitotic activity. All this evidence indicates that seedlings with a normal mitotic index were more sensitive to radiation than those in which the number of dividing cells was reduced by DNC treatment.

Chart 3 was constructed on the basis of Exp. 3 in the series recorded in Table 4. This table shows

that when the two groups of seedlings were treated with identical doses of HN2, the group with a normal rate of cell division was only slightly more sensitive than the group with cell division reduced by DNC. This difference is not significant, since $0.01 < P < 0.02$. While all the roots had laterals at the end of the experiments, the normal cell division group showed a slightly increased sensitivity to HN2, as indicated by the laterals in these seedlings appearing somewhat later than in the group with the reduced cell division.

DISCUSSION

A comparison of the "cell division present" columns in Tables 1 and 2 shows that, after the use of these doses of radiation or HN2, the percentages of roots which stopped growing were nearly the same. The average length of the primary roots at the time of death and the number of seedlings in which lateral root growth was entirely suppressed were approximately equal in both groups. Since about the same amount of injury resulted from the use of the two agents, the doses of x-rays and HN2 used in these experiments with 16-hour germinated embryos are biologically equivalent to one another. However, if younger embryos containing no dividing cells were used as the test material, with the same dose of HN2, a considerably larger dose of x-rays would be required to kill the same percentage of roots.

It will be noted in Table 1 that 3,500 and 4,000 r were used to stop the growth of 73 per cent of the 16-hour germinated embryos, but the results of Table 3 show that only 1,700 and 1,750 r were required to produce the same mortality in the group with the normal rate of mitosis. The greater sensitivity of the older seedlings as shown in Table 3 may be due, in part, to the increase in hydration and general metabolic rate, but it is probable that most of the increased sensitivity can be attributed to the higher mitotic rate.

One obvious reason for the effectiveness of the more dilute solution of HN2 used to treat the older seedlings (Table 4) is that these roots could ab-

TABLE 1

COMPARISON OF THE EFFECT OF X-RAYS ON CORN EMBRYOS CONTAINING DIVIDING CELLS AND YOUNGER EMBRYOS WITH NO DIVIDING CELLS AT THE TIME OF RADIATION

		Cell division present (15 per cent)					Cell division absent				
			Controls		Treated			Controls		Treated	
Exp. no.	X-ray dose	Germination time (hr.)	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing	Germination time (hr.)	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing
1	4,000 r	15	31	10	13	30	11	28	4	15	20
2	3,500 r	15			14	43	11			14	21
3	4,000 r	16	31	0	29	97	12	31	0	30	20
4	4,000 r	16	32	0	25	84	12	32	0	29	41
5	4,000 r	16	32	6	29	86	12	33	0	28	15
6	4,000 r	16	16	0	32	62	12	16	0	28	10
Total			142	3.5*	142	73		140	0.7*	144	22*

* Weighted averages.

TABLE 2

COMPARISON OF THE EFFECTS OF HN2 ON CORN EMBRYOS CONTAINING DIVIDING CELLS AND YOUNGER EMBRYOS WITH NO DIVIDING CELLS AT THE TIME OF TREATMENT

		Cell division present (15 per cent)					Cell division absent				
			Controls		Treated			Controls		Treated	
Exp. no.	HN2 dose 5.2×10^{-5} M (min.)	Germination time (hr.)	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing	Germination time (hr.)	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing
1	30	15	29	0	29	45	11	20	10	28	78
2	30	15	36	0	36	55	11	36	0	33	39
3	30	16	38	0	33	55	12	35	0	30	56
4	30	16	33	0	32	62	12	34	0	72	46
5	30	15	31	0	53	75	11	31	0	41	63
6*	30	16	18	0	70	97	12	17	0	69	91
Total			182	0	253	71†		173	1.2†	273	62†

* pH of water higher, HN2 solution pH 6.6.
† Weighted averages.

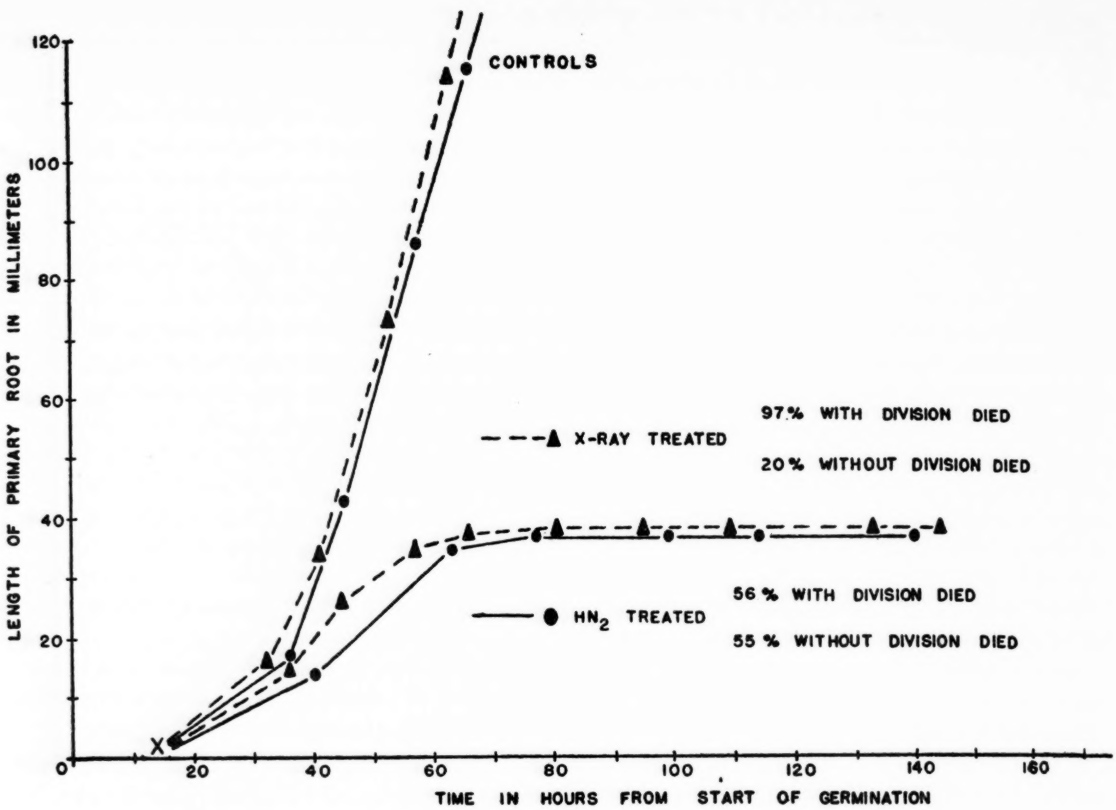


CHART 1.—Growth curves of primary roots of corn seedlings which died after exposure to 4,000 r and 5.2×10^{-5} M HN_2 solution for 30 minutes, respectively.

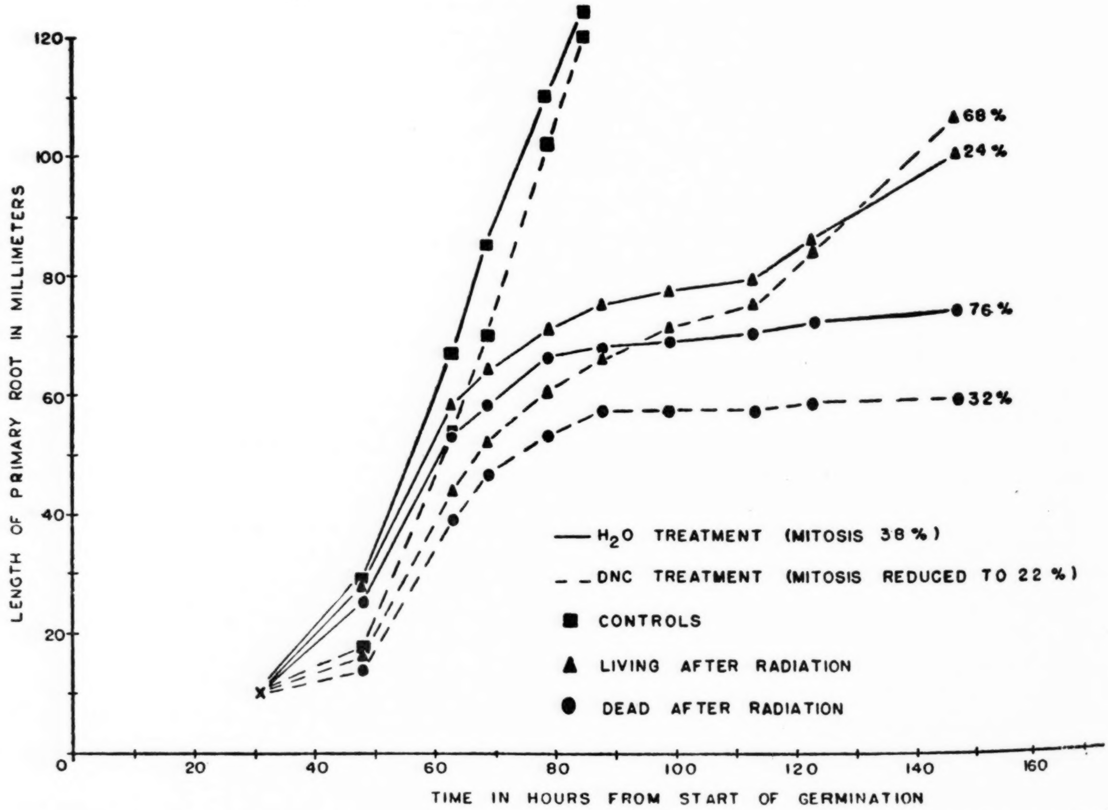


CHART 2.—Growth curves of primary roots of seedlings radiated with 1,700 r after soaking in water (38 per cent mitosis) and after soaking in DNC (22 per cent mitosis).

sorb the solution freely, while the roots of all the younger embryos (Table 2) were protected by the intact pericarp.

The embryos of the two age groups used in Method A differed from one another not only in the number of dividing cells, but also in that the tissue of the older group was more highly differentiated and had a greater degree of hydration. The last two variables were eliminated in Method B, which made use of seedlings of the same age. When DNC seedlings were radiated, they were less sensitive than normal seedlings. If the respiration rate had been increased by the DNC pretreatment, one would expect sensitivity to increase also. This tendency may have been present and its effect masked by the effect of decreasing rate of mitosis on lowering sensitivity. The possibility of any interaction between DNC and HN2 would seem to be ruled out by the results of the following experiment: when HN2 was dissolved in a 10^{-5} M

TABLE 3

COMPARISON OF THE EFFECT OF X-RAYS ON NORMAL CORN SEEDLINGS WITH 38 PER CENT DIVIDING CELLS AND ON SEEDLINGS TREATED WITH DNC TO REDUCE CELL DIVISION TO 22 PER CENT

		Normal cell division (38 per cent)				Cell division reduced to 22 per cent by DNC			
		Controls		Treated		Controls		Treated	
Exp. no.	X-ray dose	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing
1	1,750 r	34	0	39	69	26	4	43	32
2	1,700 r	16	0	34	76	20	0	38	34
3	1,700 r	18	0	38	76	17	0	37	32
4	1,700 r	15	0	30	66	16	0	35	25
Total		83	0	141	72*	179	1*	153	31*

* Weighted averages.

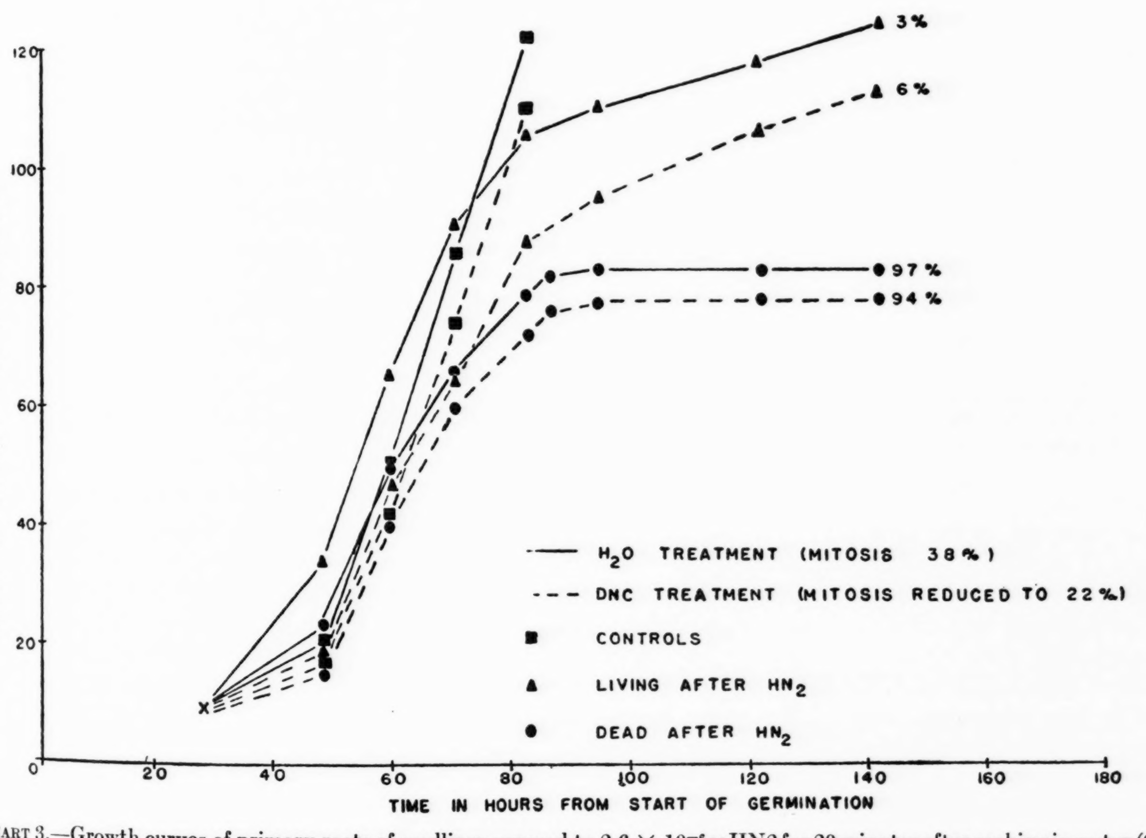


CHART 3.—Growth curves of primary roots of seedlings exposed to 2.6×10^{-6} M HN2 for 20 minutes after soaking in water (38 per cent mitosis) and after soaking in DNC (22 per cent).

solution of DNC instead of water, seedlings exposed to this solution behaved the same as seedlings treated with the same concentration of HN2 made up in water.

Table 5 summarizes the data presented in the other tables. The mortality figures in line 2 show that, with the use of either method, corn root tissue with the greater amount of cell division present at the time of radiation was much more sensitive to x-rays than the same tissue with less cell division. In line 3 the figures from both methods show that, when seedlings were exposed to HN2, the variation in the amount of cell division at the time of exposure made no significant difference in the sensitivity of the roots. These results show that under the conditions of these experiments differences in mitotic activity during exposure affected sensitivity to HN2 to a negligible degree, while the radiosensitivity of the same tissue increased with the amount of cell division present at the time of exposure to x-rays.

The data indicating a marked rise in sensitivity of embryo roots to radiation, at about the time that mitosis commences, agree with the findings of Henshaw and Francis (4), who reported similar results with wheat seedlings. The fact that the roots whose rate of cell division was reduced by DNC were less sensitive to x-rays than normal roots of the same age is also in agreement with the general conception that sensitivity to radiation is usually directly proportional to the mitotic index of the tissue.

The experimental data presented here confirm the statement by Strong (7) that nitrogen mustard seemed to affect cells even before there was any evidence of cell division. It may be that, although the seedlings showed the effects of x-rays and HN2 in many similar ways, the action of the two agents was different when the reaction of resting cells was compared to that of dividing cells. Further experimentation may show whether this difference is mainly in the initial damage or whether the dividing cells are better able to recover after exposure to HN2 than after x-ray treatment.

TABLE 4

COMPARISON OF THE EFFECT OF HN2 ON NORMAL CORN SEEDLINGS AND ON SEEDLINGS TREATED WITH DNC TO REDUCE CELL DIVISION

		Normal cell division (38 per cent)				Cell division reduced to 22 per cent by DNC			
		Controls		Treated		Controls		Treated	
Exp. no.	HN2 dose 2.6×10^{-6} M (min.)	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing	No. of roots	Per cent roots stopped growing
1	16	17	0	46	17	17	0	57	8
2	18	18	0	30	63	17	0	30	46
3*	20	19	0	69	97	18	0	71	94
4	20	33	6	84	32	34	0	85	29
Total		88	2†	229	53†	86	0	243	46†

* pH of water higher, HN2 solution pH 6.6.
† Weighted averages.

TABLE 5

COMPARISON OF THE EFFECTS OF X-RAYS OR HN2 WHEN APPLIED TO TISSUE WITH VARYING AMOUNTS OF CELL DIVISION OBTAINED BY TWO DIFERENT METHODS

	Method A (Tables 1 and 2)				Method B (Tables 3 and 4)			
	Per cent mitosis at time of treatment							
	15		0		38		22	
	15- or 16-hr. germinated embryos		11- or 12-hr. germinated embryos		Normal 48-hr. seedlings		DNC-treated 48-hr. seedlings	
	No. of plants	Per cent mortality	No. of plants	Per cent mortality	No. of plants	Per cent mortality	No. of plants	Per cent mortality
Controls for radiation	142	3.5	140	0.7	83	0	179	1
Treated with X-rays	142	73.0	144	22.0	141	72	153	31
Treated with HN2	253	71.0	273	62.0	229	53	243	46
Controls for HN2	182	0	173	1.2	88	2	86	0

SUMMARY

1. Two methods were used to obtain corn seedlings with little or no cell division for comparison

with similar tissue containing a higher percentage of dividing cells:

a) Embryos were germinated 12 hours for root tips containing no cell division and 16 hours for tips with about 15 per cent of the meristematic cells dividing.

b) Older seedlings were treated with 3,5-dinitro-*o*-cresol, which reduced the number of dividing cells in the root tip from the normal 38 per cent to 22 per cent.

2. When either method was used, the group of seedlings with the higher mitotic index was only slightly more sensitive to the effects of methyl-bis(β-chloroethyl)amine hydrochloride (HN2) than the group with the lower index.

3. When either method was used, the group of seedlings which had the greater number of dividing cells at the time of exposure to x-rays was damaged significantly more than the group where there were fewer dividing cells.

4. Under the same experimental conditions, corn root tissue showed a significant increase in sensitivity to x-radiation with increase in the number of dividing cells, whereas no such relationship existed with the action of HN2.

5. The ratios between biologically equivalent doses of x-rays and a mustard compound may vary widely for the same tissue if its mitotic index is raised or lowered.

ACKNOWLEDGMENTS

We wish to acknowledge the courtesy of the Delaware Hospital which furnished the radiation.

REFERENCES

1. CLOWES, G. H. A., and KRAHL, M. E. Action of Dinitro Compounds on Sea Urchin Eggs. Science, **80**:384–85, 1934.
2. FRIEDENWALD, J. S., and BUSCHKE, W. Studies on the Physiology, Biochemistry, and Cytopathology of the Cornea in Relation to Injury by Mustard Gas and Allied Toxic Agents. V. Nuclear Fragmentation Produced by Mustard and Nitrogen Mustards in the Corneal Epithelium. Bull. Johns Hopkins Hospital, **82**:161–78, 1948.
3. GILMAN, A., and PHILIPS, F. S. The Biological Actions and Therapeutic Applications of the β-Chloroethyl Amines and Sulfides. Science, **103**:409–15, 1946.
4. HENSHAW, P. S., and FRANCIS, D. S. A Consideration of the Biological Factors Influencing the Radiosensitivity of Cells. J. Cell. & Comp. Physiol., **7**:173–95, 1935.
5. RUSSELL, M. A. Effects of X-Rays on Zea Mays. Plant Physiol., **12**:117–33, 1937.
6. ———. Nitrogen Mustard and X-Ray Research, pp. 1–7. Research Reviews (Office of Naval Research), May, 1950.
7. STRONG, L. C. Induction of Mutations by a Carcinogen. pp. 486–99. Proc. 8th Internat. Cong. of Gen., 1948.

The Effect of Neoplasia or Pregnancy on the Tissue Desoxypentosenucleic Acid*

LOLA S. KELLY, ANITA H. PAYNE, MARGARET R. WHITE, AND HARDIN B. JONES

(*Donner Laboratory of Medical Physics, University of California, Berkeley, Calif.*)

In an earlier paper (1), a preliminary report was made of the increased phosphate turnover of desoxypentosenucleic acid in the tissues of mice bearing a transplanted mammary carcinoma. The same effect has since been found in Slonaker rats bearing transplants of lymphosarcoma and in pregnant mice and rats.

The relative nucleic acid turnover rate was measured by giving a tracer dose of radioactive sodium phosphate intraperitoneally, sacrificing the animals after a given time interval, isolating the desoxypentosenucleic acid from the tissues to be investigated, and measuring the specific activity of the nucleic acid phosphorus.

The method used for the isolation of the desoxypentosenucleic acid was Levene's, except for some changes which had been found necessary in order to make it suitable for a tracer experiment. The details of the method were given in an earlier paper (1).

* From the Division of Medical Physics and the Radiation Laboratory, Department of Physics.

Received for publication March 24, 1951.

The measurements on A strain mice bearing bilateral transplants of Strong's mammary carcinoma were extended in an effort to determine the relationship between the age or size of the tumor and the nucleic acid turnover rate of the tissues. The results are given in Table 1 and Chart 1. In all the experiments the mice used were females, weighing between 21 and 25 gm., and they were sacrificed precisely 2 hours after the administration of the tracer sodium phosphate. All animals were sacrificed at 1 P.M., since we had found previously that a marked diurnal variation exists.

TABLE 1

AVERAGES OF MOUSE DESOXYPENTOSENUCLEIC ACID SPECIFIC ACTIVITIES × 10⁴*

Tumor age (days)	Tumor weight (gm.)	No. of animals	Liver	Spleen	Kidney	Small intestines
0 (Controls)		264	1.01±0.1	36.9±1.5	0.54±0.05	15.3±0.6
5–10	0.084–0.88	144	2.42±0.15	59.5±2.8	0.79±0.07	14.0±0.46
11–20	1.6 –4.1	144	3.80±0.22	64.0±2.8	0.87±0.05	15.0±0.7
5	0.084	24	2.10±0.32	57.8±3.8	0.93±0.13	17.5±0.46
7	0.37	24	1.56±0.14	66.0±9.0	0.51±0.25	12.1±0.16
8	0.36	24	2.66±0.17	33.9±3.7	0.73±0.06	13.9±0.16
8	0.52	24	3.36±0.43	85.5±5.0	0.81±0.08	14.9±0.50
9	0.17†	24	2.67±0.17	60.9±3.6	1.37±0.06	17.2±1.1
10	0.88	24	1.81±0.5	58.3±3.0	0.42±0.12	8.1±0.51
11	1.6	24	2.93±0.29	71.0±1.9	1.09±0.10	18.3±1.3
11	1.96	24	2.80±0.23	72.0±5.0	0.67±0.04	10.8±0.6
14	2.7	24	3.17±0.32	46.6±3.5	0.81±0.10	14.2±1.2
15	2.8	24	4.16±0.49	67.5±1.8	0.93±0.14	9.6±0.4
15	2.9	24	4.57±0.56		1.04±0.10	14.3±1.6
20	4.1	24	5.4 ±0.7	63.0±9.0	0.65±0.11	23.1±1.1
Pregnant		12	2.58±0.6	82.0±16	3.8 ±1.3	20.0±1.7

* The values given represent the number of P^{32} counts per milligram of phosphorus divided by the number of counts injected normalized for a 25-gm. mouse weight.

† For some reason unknown to the authors the tumors in an occasional group of transplants grow more slowly than usual.

As is evident from the data in Table 1 only the livers, among the tissues investigated, show a clear relation between the age of the carcinoma transplant and the nucleic acid specific activity. The specific activity of the spleens and kidneys is higher for all the tumor groups than for the controls, but the data are not sufficient to indicate whether there is a tumor-age dependency. The average small intestine nucleic acid specific activity of all the tumor animals is the same as that for the control group, although in some of the experiments

the variations are somewhat larger than one might expect. The standard errors quoted are based only on the internal consistency of each experiment. There are undoubtedly additional unknown variables between individual experiments.

Table 1 also includes data on a small group of pregnant mice in the third week of gestation. The average weight of the fetuses was 6.3 gm. per mouse. This very large amount of fetal tissue introduces a considerable uncertainty into our measurement of the nucleic acid turnover. It may divert a disproportionately large amount of the tracer phosphate from the mother and thus cause a lower specific activity than would otherwise be the case. However, it is felt that this group does show a significantly increased turnover rate in the livers, spleens, and kidneys. The values for the small intestines show too much overlapping with the normal group to be decisive.

Table 2 summarizes the results in a series of experiments on Slonaker rats. The general procedures were the same as those used in the mouse experiments, except that the animals were sacrificed 4 hours after the administration of the tracer phosphate. The average tracer dose was 50 μc. The desoxypentosenucleic acid was isolated separately from each liver and small intestine, while the spleens and kidneys from several animals were pooled.

The tumors used were bilaterally transplanted lymphosarcomas, which in our experience do not metastasize. The average weight of tumor tissue per rat was 13.2 gm. These animals again showed the marked increase in the specific activity of the liver, spleen, and kidney desoxypentosenucleic acids and the apparently normal specific activity of the small intestine nucleic acid.

The pregnant rats measured were in the second week of gestation, and the average weight of fetal tissue per rat was 4 gm. This is only 2 per cent of the rat's body weight and hence could not affect the amount of tracer phosphate available to the mother very materially. In this group only the liver and spleen nucleic acids were measured, but they show a much higher specific activity than the controls.

In a series of six experiments an attempt was made to find a humoral agent in the serum of five cancer patients and one pregnant woman which would raise the nucleic acid turnover rate of mice when the serum was injected. The following procedure was used: 1 cc. of freshly prepared serum from a patient was injected subcutaneously into each of twelve mice. Two hours later 1 more cc. of serum was given subcutaneously, and at the same time the tracer dose of phosphate was given intraperitoneally. The mice were then sacrificed and the specific activity of the liver, spleen, and kidney nucleic acids determined as usual. Six control groups using serum from normal individuals were also measured. In none of the above experiments did the average nucleic acid specific activity differ significantly from untreated controls.

In one further serum experiment 24 mice were injected with serum from 24 other mice bearing bilateral transplants of mammary carcinoma weighing approximately 1 gm. Serum in the amount of 0.2 cc. was injected intravenously, and 2 hours later 0.15 cc. more serum was given intravenously. At the same time, the tracer phosphate was in-

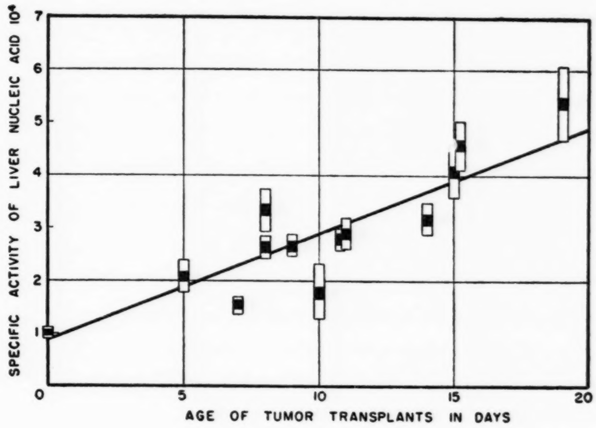


CHART 1.—The relationship between age of tumor and specific activity of liver desoxypentosenucleic acid in mice bearing mammary carcinomas.

TABLE 2

AVERAGES OF RAT DESOXYPENTOSENUCLEIC ACID SPECIFIC ACTIVITIES $\times 10^5$

	No. of animals	Liver	Spleen	Kidney	Small intestines
Controls	28	2.25±0.14	73.3± 5.5	1.2±0.2	81.5±3.4
Lymphosarcoma†	32	6.27±0.97	126.0± 7.0	2.2±0.2	88.5±4.5
Pregnant‡	13	[illegible].52±0.37	212.0±12.0		

* The values given represent the number of P^{32} counts per milligram of phosphorus divided by the number of counts injected normalized for a 200-gm. rat weight.

† Average weight of tumor tissue, 13.2 gm.

‡ Average weight of fetal tissue, 4.0 gm.

jected intraperitoneally. The animals were sacrificed 2 hours later and treated as usual. No significant difference was observed between the specific activities of these animals and control groups.

A series of preliminary experiments was also done with crude tumor and embryonic extracts in our attempt to reproduce the effects of the intact tissues. However, the results have been inconclusive thus far.

The increased specific activity of the nucleic acid in the tissues of pregnant and tumorous animals might be owing either to a purely metabolic effect, caused by the increased demands on the organism for tissue synthesis, or to some specific humoral agent liberated by the rapidly dividing tissues.

Experiments are now in progress to seek an explanation of the observed phenomena.

SUMMARY

The specific activity of desoxypentosenucleic acid was measured in the liver, spleen, kidneys, and small intestines of (*a*) A strain mice bearing bilateral transplants of Strong's mammary carcinoma, (*b*) pregnant mice, (*c*) Slonaker rats with bilateral lymphosarcomas. It was also measured in the liver and spleen of pregnant rats.

In all groups investigated the specific activity of the desoxypentosenucleic acid of the liver, spleen, and kidneys was found to be higher than that of the controls. In the small intestines no appreciable rise was noted.

In the mice bearing mammary carcinoma the specific activity of the desoxypentosenucleic acid of the liver shows a clear relationship to the age of the tumor.

Inconclusive attempts were made to find some agent which might cause this rise in specific activity of DNA.

REFERENCES

1. Kelly, L. S., and Jones, H. B. Effect of Neoplastic Tissue on the Turnover of Desoxypentose Nucleic Acid. Science, **111**:333–34, 1950.

Effect of Anti-viral Substances on the Mouse Mammary Tumor Milk Agent *in Vivo*

R. A. Malmgren and L. W. Law

(*National Cancer Institute, Bethesda 14, Md.*)*

INTRODUCTION

The mouse mammary tumor milk agent, which has been shown to play a principal part in the genesis of some mouse mammary tumors (3), has the physical, chemical, and biological properties of a virus (1, 2, 7). The virus-like nature of the milk agent suggests that it may be inhibited by some of the recently discovered anti-viral substances.

Inactivation of the agent *in vivo* without injury to the mouse would permit a better understanding of the function of the milk agent in the production of mammary tumors and would help to determine whether or not the virus is necessary as a continuing cause of cancer.

Four viricidal compounds were tested for their effect on the milk agent. The compounds chosen were aureomycin,[1] chloramphenicol, and streptomycin, which have been used successfully in experimental and clinical rickettsial and lymphogranuloma venereum infections (10–12, 14, 16), and sodium phenosulfazole (Darvisul) which has been reported (13), though not confirmed (5, 9, 15), as being effective against mouse poliomyelitis virus. In addition, it has been shown that chloramphenicol is effective in inactivating a gene-controlled substance, "Kappa," in Paramecium (4). This is of particular interest, since, as shown by Heston *et al.* (8), the concept of gene-cytoplasm relationship is strikingly applicable to the mammary tumor problem.

Data presented in Table 1 indicate the effect of these four antibiotics on the mouse mammary tumor milk agent *in vivo*.

MATERIALS AND METHODS

Two groups of animals were used: Group I, consisting of C3H[2] female mice of the F15 and F16 generations, 4 weeks old; and Group II, consisting of strain C (B alb C)[3] female mice, 3 weeks old. All the treated mice received the approximate maximum tolerated dose which had been previously determined for each compound.

Group I animals, C3H mice which had obtained the milk agent from their mothers, were given the test material for 7 days, beginning at 4 weeks of age. These mice were maintained as virgins and were observed for the effect of the antibiotic on the tumor incidence and mean tumor age. Group II animals, C mice presumably without the milk agent, were treated for 48 hours with the anti-viral compound and then injected intraperitoneally with 0.2 cc. of a spontaneous C3H mammary tumor filtrate (Berkefeld) containing the milk agent. The filtrate was prepared from 1 part tumor and 5 parts of Locke's solution. This was followed by 5 more days of treatment with the substance being tested. The C mice were force-bred, and the tumor incidence and mean tumor age were noted.

All the antibiotics were administered subcutaneously 2 times a day, with the exception of sodium phenosulfazole, which was injected intraperitoneally 6 times a day (one injection every 4 hours). One-tenth cc. of each antibiotic was given at each injection. Aureomycin was dissolved in distilled water so that each 0.1 cc. contained 0.5 mg. Chloramphenicol was dissolved in 0.6 per cent propylene glycol, and each 0.1 cc. contained 0.4 mg. of the antibiotic. Streptomycin was made up in distilled water so that each 0.1 cc. contained 3,000 units. Sodium phenosulfazole was dissolved in a phosphate buffer of pH 8.6, and each 0.1 cc. contained 4.0 mg. of the compound.

Control mice received the same number of injections at the same time as the treated animals, but in the controls only the solvent was administered.

* National Institutes of Health, Public Health Service, Federal Security Agency.

[1] Aureomycin and sodium phenosulfazole were kindly supplied by Lederle Laboratories Division of the American Cyanamid Co., Pearl River, N.Y., and chloramphenicol by Parke, Davis & Co., Detroit, Mich.

Received for publication April 25, 1951.

[2] Strain C3H of the National Cancer Institute subline. These were obtained originally from Dr. W. E. Heston of this Institute.

[3] The spontaneous mammary tumor incidence of strain C breeding females has been found to be less than 1 per cent in our colony.

In Group I, litter-mates were used as controls. In Group II, mice born at approximately the same time, though not necessarily litter-mates, were used as controls.

RESULTS AND DISCUSSION

None of the antibiotics tested produced statistically significant differences in the tumor incidence between treated and control mice. Control mice were maintained separately for each antibiotic. In the table, however, the controls are grouped, since the tumor incidence and the mean tumor age were nearly identical for all groups. Animals which died before 6 months of age were not included in the analysis. In the case of Group II, sodium phenosulfazole-treated animals, where only 11 out of 30 animals survived beyond 6 months, statistical analysis is of little value. Since the age at death of the nontumorous animals in the sodium phenosulfazole-treated Group II is lower than the mean tumor age for this group, it is obvious that some of the animals which died early without tumors might have developed tumors had they lived longer. When this is considered along with the apparent absence of effect of sodium phenosulfazole in the Group I animals, it is doubtful whether the decreased percentage of mammary tumors in sodium phenosulfazole Group II is of significance.

The mean tumor age was not statistically different for the control and treated animals in Groups I and II, with the exception of the animals in Group II which received streptomycin. In this group, treated with streptomycin, 86.6 per cent of the tumors which ultimately arose appeared in the first 11 months; however, only 35.7 per cent of the tumors which developed in the control mice had appeared at this time. The mean tumor age for the treated mice in this group was 9.8 ± 0.67 months, while for the controls it was 13.5 ± 1.22 months. The difference of the mean is, therefore, 3.7 ± 1.22, where $P < 0.01$. This places the difference on the border line of significance. Since the streptomycin-treated and control mice in Group I show no significant difference in mean tumor age, the effect noted in Group II may represent a sampling error. Further study is perhaps indicated, however, since this may suggest an "activating effect" of streptomycin on the milk agent. A similar phenomenon has been previously noted on *T. pallidum* (6).

These results would indicate that the milk agent was not affected by aureomycin, chloramphenicol, or sodium phenosulfazole, when exposed to them in the manner described, and that streptomycin was without effect, except possibly in shortening the mean tumor age in C mice.

TABLE 1

THE EFFECT OF ANTI-VIRAL SUBSTANCES ON THE MAMMARY TUMOR INCIDENCE AND MEAN TUMOR AGE IN STRAIN C AND C3H MICE

Group	Antiobiotic tested	Dosage Daily	Dosage Total	Mouse strain	No. of mice	Mammary tumors No.	Mammary tumors Per cent	Mean age tumor (months)	Mean age at death non-tumorous (months)
I	Aureomycin	2×0.5 mg.	7 mg.	C3H	20	20	100	10.0	
	Streptomycin	2×3,000 units	42,000 units		19	19	100	9.0	
	Chloramphenicol	2×0.4 mg.	5.6 mg.		20	20	100	9.2	
	Sodium phenosulfazole	6×4.0 mg.	168 mg.		16	16	100	9.2	
	Controls				71	71	100	9.7	
II	Aureomycin	2×0.5 mg.	7 mg.	B alb C	31	17	55	14.7	16.4
	Streptomycin	2×3,000 units	42,000 units		32	15	47	9.8	18.2
	Chloramphenicol	2×0.4 mg.	5.6 mg.		20	10	50	11.0	17.0
	Sodium phenosulfazole	6×4.0 mg.	168 mg.		11	4	27	14.0	13.5
	Controls				79	36	45	12.7	18.5

SUMMARY

The effect of several anti-viral agents was studied in C3H mice containing the mammary tumor milk agent and in strain C mice given the agent during the course of treatment.

Aureomycin, chloramphenicol, and sodium phenosulfazole (Darvisul), when used in maximum tolerated doses, did not affect the tumor-producing properties of the milk agent.

Streptomycin did not influence the incidence of mammary tumors in the strains studied, but the mean tumor age was lowered in C mice given the mammary tumor milk agent.

REFERENCES

1. ANDERVONT, H. B. The Milk Influence in the Genesis of Mammary Tumors. A.A.A.S. No. 22, pp. 133–35. Lancaster: Science Printing Co., 1945.
2. BARNUM, C. P., and HUSEBY, R. A. The Chemical and Physical Characteristics of Preparations Containing the Milk Agent Virus: *A Review*. Cancer Research, 10:523–29, 1950.
3. BITTNER, J. J. Some Possible Effects of Nursing on the

Mammary Gland Tumor Incidence in Mice. Science, **84:**162, 1936.

4. BROWN, C. H. Nature of "Kappa" Particles in Paramecium. Brit. Empire Cancer Campaign, 27th Ann. Report, pp. 136–37, 1949.

5. COX, H. R.; KOPROWSKI, H.; MOYER, A. W.; SHARPLESS, G. R.; and WONG, S. C. Failure of Phenosulfazole (Darvisul) in Treating Experimental Viral Infections. Proc. Soc. Exper. Biol. & Med., **70:**530–34, 1949.

6. FISKEN, R. A., and GRUHZIT, O. M. The Effect of Streptomycin on Experimental Syphilis Infection of Rabbits. Am. J. Syph. & Gonor. & V.D., **30:**581–85, 1946.

7. GRAFF, S.; MOORE, D. H.; STANLEY, W. M.; RANDALL, H. T.; and HAAGENSEN, C. D. Isolation of Mouse Mammary Carcinoma Virus. Cancer, **2:**755–62, 1949.

8. HESTON, W. E.; DERINGER, M. K.; and ANDERVONT, H. B. Gene-Milk Agent Relationship in Mammary-Tumor Development. J. Nat. Cancer Inst., **5:**289–307, 1945.

9. LO GRIPPO, G. A.; EARLE, D. P., JR.; BRODIE, B. B.; GRAEF, I. P.; BOWMAN, R. L.; and WARD, R. Lack of Effect of Sodium Phenosulfazole (Darvisul) on Certain Experimental Virus Infections. Proc. Soc. Exper. Biol. & Med., **70:**528–29, 1949.

10. MCLEAN, I. W., JR.; SCHWAB, J. L.; HILLEGAS, A. B.; and SCHLINGMAN, A. S. Susceptibility of Micro-organisms to Chloramphenicol (Chloromycetin). J. Clin. Investigation, **28:**953–63, 1949.

11. RAKE, G.; PANSY, F.; JAMBOR, W. P.; and DONOVICK, R. Investigations on Streptomycins. J. Clin. Investigation, **28:**846–49, 1949.

12. ROSCOVE, L.; WEST, H. E.; and BOWER, A. G. Q-Fever: Case Treated with Streptomycin. Ann. Int. Med., **28:** 1187–92, 1948.

13. SANDERS, M.; SUBBAROW, Y.; and ALEXANDER, R. C. An Effective Anti-viral Synthetic. Texas Rep. Biol. & Med., **6:**385–95, 1948.

14. SMADEL, J. E.; WOODWARD, T. E.; LEY, H. L.; PHILIP, C. B.; TRAUB, R.; LEWTHWAITE, R.; and SAVOOR, S. R. Chloromycetin in the Treatment of Scrub Typhus. Science, **108:**160–61, 1948.

15. WEIL, M. L.; and WARREN, J. Therapeutic Failure of Phenosulfazole (Darvisul) in Mice Infected with EMC or with MM Viruses. Proc. Soc. Exper. Biol. & Med., **70:** 534–35, 1949.

16. WRIGHT, L. T.; SANDERS, M.; LOGAN, M. A., PRIGOT, A.; and HILL, L. M. Aureomycin: A New Antibiotic with Virucidal Properties. J.A.M.A., **138:**408–12, 1948.

Sterilization of Leukemic Cells *in Vivo* and *in Vitro**

J. H. Burchenal,† M. A. Cremer, B. S. Williams, and R. A. Armstrong

(*Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, N.Y.*)

Certain 4-amino derivatives of pteroylglutamic acid (PGA), as well as various nitrogen mustards and ethylenimines, have shown chemotherapeutic activity in screening tests against transplanted mouse leukemia (3–7, 12, 14, 15). In an attempt to differentiate between the modes of action of these compounds, a technic has been employed (4, 11) which evaluates the ability of compounds to affect *in vivo* the transplantability of leukemic cells.

A somewhat similar technic has been employed previously to test the *in vivo* effect of certain agents against tumor cells. Sugiura (16–18) demonstrated that irradiation has an inactivating effect *in vivo* on Sarcoma 180 in the mouse. Karnofsky (11) has shown a similar effect with x-ray and methyl-bis(2-chloroethyl)amine (HN2) on Sarcoma 180 when grown on the chlorioallantoic membrane of the developing chick embryo, whereas the 4-amino derivatives of PGA tested by the same technic had no effect.[1]

Results of preliminary experiments on the ability of a nitrogen mustard and two 4-amino derivatives of PGA to inactivate leukemic cells *in vivo* showed that HN2 had a definite sterilizing effect, while the two derivatives of PGA were without this effect (4). Although the 4-amino derivatives of PGA were demonstrated by Biesele (1) to be inactive in tissue culture, 2,6-diaminopurine, an adenine antagonist (10), damaged mouse sarcoma cells in this technic at concentrations which were well tolerated by normal mouse tissue (1).

On the basis of these preliminary results, six nitrogen mustards, two ethylenimines, five antagonists of PGA, and 2,6-diaminopurine were evaluated for their ability to sterilize leukemic cells *in vivo* and so prevent the transmission of the disease on bioassay into normal mice. Similar studies were done with 4-amino-N^{10}-methyl-PGA in bilaterally nephrectomized leukemic mice. The *in vitro* effects of some of these compounds were also investigated.

METHOD

Leukemias Ak4 (2) and Ak 9417 (7), both lymphoid strains, and chloroleukemia Ak 1394 (8) were used in these studies. Mice which received subcutaneous injections of Ak 1394 and Ak 9417 developed tumors at the site of injection. Mice inoculated with Ak4 leukemia by the same route, however, developed a generalized form of the disease without tumor formation, and for this reason intraperitoneal injections were used with this strain. Six- to 8-week-old mice of the inbred Akm stock were used in these experiments. Between 8 and 14 days after the transplantation of the leukemia, manifestations of the disease were present in all three strains, and at this time the animals were injected intraperitoneally with the compound to be tested. The doses used were usually started at the acute LD_{50} and were increased by increments of 100 per cent with two donor mice used at each dosage level. The acute LD_{50} dose was determined on the basis of preliminary toxicity studies conducted by Dr. Frederick S. Philips of the Department of Pharmacology, Sloan-Kettering Institute. Except as otherwise noted the donor mice were sacrificed 2 hours after injection, and a suspension of tumor or spleen was made in physiological saline. Four recipient mice of the same age and stock as the donor animal received intraperitoneal inoculations of 0.1 cc. each of the suspensions of tumor or spleen. If the donor mice died within 2 hours, the transfers were made at the time of death. The recipient mice were examined at autopsy for gross manifestations of leukemia, and if evidence was not conclusive, microscopic sections were taken. In this technic advantage was taken of the fact that many of the compounds tested showed delayed toxicities, even at supralethal doses. Animals receiving injections of PGA antagonists at dosage levels up to 100 times the LD_{50} usually survived for 2–3 days. It was therefore possible to leave the compound in the body of the donor animal from 24 to 72 hours before transfer of the leukemia.

* This investigation was supported, in part, by a research grant from the National Cancer Institute of the National Institute of Health, United States Public Health Service, and, in part, by a research grant from the American Cancer Society.

† Damon Runyon Senior Clinical Research Fellow.

[1] D. A. Karnofsky, personal communication.

Received for publication April 26, 1951.

Another technic developed for this study required a series of bilaterally nephrectomized leukemic animals as donors. Half an hour after nephrectomy, doses of 4-amino-N^{10}-methyl-PGA, varying from 750 to 3,000 mg/kg were administered intraperitoneally to a series of these mice. Immediately after the animals died, the spleens were removed, washed, minced in physiological saline and transferred to recipient mice, by the same technic employed in the other *in vivo* inactivating experiments. A series of sham-operated controls was run at the same time and transferred in the same manner. Unfortunately, after nephrectomy and the injection of these large doses of drug, the survival time of these sick donor mice was only from 13 to 19 hours.

An *in vitro* technic was also employed with leukemia Ak4. In this test, solutions of the compound at various concentrations were added to a suspension of leukemic cells in physiological saline. The mixture was allowed to stand at room temperature for 1 hour, and then 0.1 cc. of the suspension containing approximately 1,000,000 cells was injected intraperitoneally into ten mice. The recipient mice were examined at autopsy for evidence of leukemia.

RESULTS AND DISCUSSION

Tables 1, 2, and 3 present the results obtained with the nitrogen mustards when tested against the three strains of leukemia. The letters listed beside the compounds in the various tables indicate the source from which the agents were supplied for these studies.[2] All the nitrogen mustards listed had been found effective previously in routine survival time experiments (3, 7). As can be seen from the tables, all the nitrogen mustards behaved in a manner similar to that of HN2. The minimum dose of most of these compounds required to prevent transmission of leukemia varied from 2 to 10 times the LD_{50}.

With the nitrogen mustards it was observed that the survival time of the recipient mice appeared to increase as levels approaching the inactivating doses were reached. It is possible that most of the leukemic cells were inactivated in the donor animal by these compounds, thereby delaying death of the recipients.

Two ethylenimine compounds (6) were tested for their inactivating effect (Table 4). 2,4,6-Tri-ethylenimino-*s*-triazine (triethylene melamine) had an inactivating effect at approximately $3\frac{1}{2}$ times the LD_{50}. A monoethylenimine, 2-ethylenimino-4,6-dimethoxy-*s*-triazine, had only a slight inactivating effect at doses up to 12 times the LD_{50}. The low activity of this latter compound can be explained on the basis of its single imine ring (3, 6).

The action of the PGA antagonists (5) appeared in direct contrast to that of the nitrogen mustards, since they showed no inactivating effect against leukemia Ak4 or Ak 9417 (Tables 5 and 6) with doses as high as 100 times the LD_{50}. All these compounds had previously demonstrated activity in prolonging the survival time of leukemic mice in preliminary screening tests and were more effective than HN2 against leukemia Ak4 (5).

Since the PGA antagonists showed a delayed toxicity even at very high doses (9), some were tested after intervals of 24, 48, and 72 hours in addition to the customary 2 hours. Despite this, in no test was there any evidence of an inactivating effect on the leukemic cells. Seventy-two hours after the injection of 25 and 50 times the LD_{50} of 4-amino-N^{10}-methyl-PGA, the spleens had decreased in size, the bone marrow showed almost complete destruction, and the white blood cell count had fallen from an average of 80,000 to 4,000. Splenic suspensions from these animals nevertheless were still able to transmit leukemia in recipient mice. Regardless of the dose used, none of the antagonists of PGA showed the tendency to lengthen the survival time of recipient mice which the nitrogen mustards and triethylene melamine had demonstrated at sub-inactivating doses. Even in nephrectomized animals whose excretion of the injected compound was inhibited, 4-amino-N^{10}-methyl-PGA at 30 times the LD_{50} for 19 hours had no inactivating effect on leukemic cells, nor did it prolong the survival time of recipient mice.

The studies of other investigators have suggested that the much greater toxicity of repeated daily injections of the 4-amino antagonists of PGA, compared to single doses, might be due to rapid excretion of these compounds (9, 13).[3] The results of the nephrectomy experiments reported here, however, seem to diminish the likelihood that the lack of an inactivating effect of the high doses of the antagonists of PGA was due to very rapid excretion through the kidney of the compound being tested.

The *in vitro* inactivation technic supplemented and provided an interesting contrast to the *in vivo* method. As might be predicted, HN2 com-

[2] We wish to thank the following groups for supplies of these compounds:

A. Lederle Laboratories.
B. Merck & Company
C. University of Chicago Toxicity Laboratory
D. Parke, Davis & Company
E. Burroughs Wellcome
F. Calco Chemical Company

[3] D. A. Karnofsky, J. B. Thiersch, D. J. Ingle, personal communication.

pletely prevented transmission of leukemia Ak4 at levels as low as 3 ml. of leukemic suspension, whereas 4-amino-N^{10}-methyl-PGA showed no *in vitro* effect at 1,000 μg/ml. This may be contrasted with the relatively similar therapeutic dosage levels, 1 and 3 mg/kg, respectively, of these compounds in the intact leukemic animal. A positive inactivating effect by this technic was of little value in itself, as there was no control over the nonspecific toxicity of the compound. When, however, no lethal action was demonstrated at high levels of the drug by this test, it assumed a

TABLE 1

IN VIVO STERILIZING EFFECT ON LEUKEMIC CELLS
LEUKEMIA AK 9417

Compound	Source	Multiples of the LD_{50} 1	2	4	8	16	32
Tris(2-chloroethyl)amine	C						
				0	0	0 0	0
LD_{50}=1.88 mg/kg		+	+	0	+	0 0	0
Methylbis(2-chloroethyl)amine hydrochloride	C	+	±				
		+	+			0	
		++	+	±00	0	000	
LD_{50}=5 mg/kg		++	+ +	000	000	000	
1,3-Propanediamine-2-chloro-N,N,N′,N′-tetrakis(2-chloroethyl)dihydrochloride	C						
			+				
		+	+ 0	0			
LD_{50}=16.7 mg/kg		++	+ 0	+0	0		
1,2-Bis(bis[2-chloroethyl]amino)-3-hydroxypropane	B						
		±		±	0	0	
LD_{50}=3 mg/kg		+	±	0	0	0	
1,2,3,4-Tetrakis(bis[2-chloroethyl]amino)butane tetrahydrochloride	B						
		±	0	0		0	
LD_{50}=50 mg/kg		+	0	0	0	0	
N,N′-Ethyl-N,N′-(bis[2-chloroethyl])-ethylenediamine dihydrochloride	B						
		+	±	+	0	0	
LD_{50}=5 mg/kg		+	+	+	+	0	

+ Recipients of donor mouse died of leukemia.
0 Recipients of donor mouse survived.
± Half of the recipients of donor died of leukemia and half survived.

TABLE 2

IN VIVO STERILIZING EFFECT ON LEUKEMIC CELLS
LEUKEMIA AK 1394

Compound	Source	Multiples of the LD_{50} 1	2	4	8	16	32
Tris(2-chloroethyl)amine	C					+	
					+ 0	0	
LD_{50}=1.88 mg/kg			+		+ 0 0	0	0
Methyl-bis(2-chloroethyl)amine hydrochloride	C				0	0	
				+	+ 0	0	
LD_{50}=5 mg/kg		+	+	+	0 0 0	0	
1,3-Propanediamine 2-chloro-N,N,N′,N′-tetrakis(2-chloroethyl) dihydrochloride	C						
				+	0		
		+	+	± 0	0 0		
LD_{50}=16.7 mg/kg		+	+ 0	0 0	0 0		
1,2,3,4-Tetrakis(bis [2-chloroethyl]amino) butane tetrahydrochloride	B						
		+	+	+			
LD_{50}=50 mg/kg		+	+	+	0 0		

+ Recipients of donor mouse died of leukemia.
0 Recipients of donor mouse survived.
± Half of the recipients of donor died of leukemia and half survived.

certain significance. 2,6-Diaminopurine lactate completely inactivated the leukemic cells at 800 μg/ml. As has been previously mentioned, similar results with mouse tumor cells in tissue culture without damage to normal cells have been noted by Biesele (1).

These technics assess the chemotherapeutic value of a compound from a slightly different aspect than does the survival time test. It is just conceivable, although rather unlikely, that by this method a compound might be discovered of which a single dose at less than LD_{50} level would

TABLE 3

IN VIVO STERILIZING EFFECT ON LEUKEMIC CELLS
LEUKEMIA AK4

Compounds	Source	Multiples of the LD_{50} 1	2	4	8	16	32	64
Methyl-bis(2-chloroethyl)amine hydrochloride	C	+		+	0			
		+		+	0			
		++	+	0+	00	00	0	0
LD_{50}=5 mg/kg		++	+	00	00	00	0	0
1,3-Propanediamine 2-chloro-N,N,N′,N′-tetrakis (2-chloroethyl) dihydrochloride	C							
		+	+	+	±			
LD_{50}=16.7 mg/kg		+	+	±	0			
1,2-Bis(bis-2-chloroethylamino)3-hydroxypropane	B		+	+	+			
			+	+	±			
		+	+	±	0	0		
LD_{50}=3 mg/kg		+	+	0	0	0		
N,N′-Ethyl-N,N′-(bis[2-chloroethyl])ethylenediamine dihydrochloride	B							
			+	+	+	0	0	
LD_{50}=5 mg/kg			+	+	±	0	0	
1,2,3,4-Tetrakis(bis[2-chloroethyl]amino)butane tetrahydrochloride	B							
		+	0	0	0	0	0	
LD_{50}=50 mg/kg		±	0	0	0	0	0	

\+ Recipients of donor mouse died of leukemia.
0 Recipients of donor mouse survived.
± Half of the recipients of donor died of leukemia and half survived.

TABLE 4

IN VIVO STERILIZING EFFECT ON LEUKEMIC CELLS
LEUKEMIA AK4

Compound	Source	Multiples of the LD_{50} 0.5	1	2	3	4	5	6	7	8	9	10	11	12
2,4,6-Triethylenimino-*s*-triazine	F													
(Triethylene melamine)		+												
LD_{50}=2.0 mg/kg		+	+	+			+							
		+	+	+			+							
		+	±	+	0		+		0					
2-hour test		0	0	+	0		+	0	0					
2,4,6-Triethylenimino-*s*-triazine	F													
(Triethylene melamine)		+	+											
LD_{50}=2.0 mg/kg		+	+											
		+ +	+	0										
24-hour test		+ +	0	0										
2-Ethylenimino-4,6-dimethoxy-*s*-triazine	F													
LD_{50}=200 mg/kg														
			+		±	+				+				
2-hour test			+	+	+	0				0		±		±

\+ Recipients of donor mouse died of leukemia.
0 Recipients of donor mouse survived.
± Half of the recipients of donor died of leukemia and half survived.

TABLE 5

IN VIVO STERILIZING EFFECT ON LEUKEMIC CELLS
LEUKEMIA AK4

Compound	Source	Multiples of the LD_{50}: 1	2	4	8	16	32	64	128	150
4-Amino-pteroylglutamic acid $LD_{50}=2$ mg/kg	D	+ +	+ +	+ +	+ +			+ +		+ +
4-Amino-pteroylglutamic acid $LD_{50}=2$ mg/kg 48-hour test	D						+ + +		+ + + +	
4-Amino-N^{10}-methyl pteroylglutamic acid $LD_{50}=50$ mg/kg	A	+ +	+ ±	++ ++	++ ++	+ +	+ +	+ +	+ +	
4-Amino-N^{10}-methyl pteroylglutamic acid $LD_{50}=50$ mg/kg 48-hour test	A						+ +		+ +	
4-Amino-N^{10}-methyl pteroylglutamic acid $LD_{50}=50$ mg/kg 72-hour test	A						+ +	+ +		
4-Amino-9-methyl pteroylglutamic acid $LD_{50}=50$ mg/kg	A						+	+ +		
4-Amino-9,10-dimethyl pteroylglutamic acid $LD_{50}=50$ mg/kg	A						+ +	+ +		
4-Amino-pteroylaspartic acid $LD_{50}=150$ mg/kg	A						+ +	+ +	+ ±	
2,6-Diaminopurine $LD_{50}=300$ mg/kg*	E	+ + +	+ +	+ + +	+ +	++ ++			+ +	

+ Recipients of donor mouse died of leukemia.
± Half of the recipients of donor died of leukemia and half survived.
* Majority of donors at and above 4 × LD_{50} survived less than one hour after injection of the compound.

TABLE 6

IN VIVO STERILIZING EFFECT ON LEUKEMIC CELLS
LEUKEMIA AK 9417

Compound	Source	Multiples of the LD_{50}: 0.5	1	2	4	8	16	32	64
4-Amino-pteroylglutamic acid $LD_{50}=2$ mg/kg	D	+ +	+ +	+	+ +	+ +			
4-Amino-pteroylglutamic acid $LD_{50}=2$ mg/kg 24-hour test	D	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
4-Amino-pteroylglutamic acid $LD_{50}=2$ mg/kg 48-hour test	D	+ +	+ +	+ +	+ +	+ +			
2,6-Diaminopurine* $LD_{50}=300$ mg/kg	E			+ +		+ +			

+ Recipients of donor mouse died of leukemia.
* Donors at 10 × LD_{50} survived less than 1 hour after injection of the compound.

have a sterilizing effect on leukemic cells. Such a hypothetical substance would be of tremendous clinical interest.

SUMMARY

1. An *in vivo* technic for inactivating leukemic cells has been described as an aid in differentiating between modes of action of compounds chemotherapeutically active against transplanted mouse leukemia.

2. Six nitrogen mustards and two ethylenimines have been tested by this method against strains Ak4, Ak 9417, and Ak 1394 of mouse leukemia. With the exception of 2-ethylenimino-4,6-dimethoxy-*s*-triazine, all showed a definite sterilizing effect on leukemic cells.

3. 2,6-Diaminopurine and five antagonists of pteroylglutamic acid were tested and found to have no inactivating properties *in vivo* at dosage levels up to 50 and 100 times the LD_{50}.

4. Even when nephrectomized mice were used in an attempt to reduce the excretion of the drug, massive doses of 4-amino-N^{10}-methyl-PGA did not sterilize the leukemic cells *in vivo*.

5. An *in vitro* technic was also described in which methylbis(2-chlorethyl)amine and 2,6-diaminopurine sterilized leukemic cells at concentrations corresponding to 1–4 times the LD_{50} dose for the intact animal. By this method, however, 4-amino-N^{10}-methyl-PGA had no effect even at concentrations corresponding to 50–100 times the LD_{50} dose.

REFERENCES

1. BIESELE, J. J. Studies on 2,6-Diaminopurine and Related Substances in Cultures of Embryonic and Sarcomatous Rodent Tissues. Cancer, **4**:186–97, 1951.
2. BURCHENAL, J. H.; BIEDLER, J. L.; NUTTING, J.; and STOBBE, G. D. The Effects of 4-Amino-N^{10}-methyl-pteroylglutamic Acid on the Leukocytes of the Normal and Leukemic Mouse. Blood, **5**:167–76, 1950.
3. BURCHENAL, J. H.; BURCHENAL, J. R.; JOHNSTON, S. F. Chemotherapy of Leukemia. III. Further Studies on the Effect of Nitrogen Mustards on Transmitted Mouse Leukemia. Cancer (in press).
4. BURCHENAL, J. H.; BURCHENAL, J. R.; KUSHIDA, M. N.; JOHNSTON, S. F.; and WILLIAMS, B. S. Studies on the Chemotherapy of Leukemia. II. The Effect of 4-Amino-pteroylglutamic Acid and 4-Amino-N^{10}-methyl-pteroylglutamic Acid in Transplanted Mouse Leukemia. Cancer, **2**:113–18, 1949.
5. BURCHENAL, J. H.; JOHNSTON, S. F.; BURCHENAL, J. R.; KUSHIDA, M. N.; ROBINSON, E.; and STOCK, C. C. Chemothrapy of Leukemia. IV. Effect of Folic Acid Derivatives on Transplanted Mouse Leukemia. Proc. Soc. Exper. Biol. & Med., **71**:381–87, 1949.
6. BURCHENAL, J. H.; JOHNSTON, S. F.; CREMER, M. A.; WEBBER, L. F.; STOCK, C. C. Chemotherapy of Leukemia. V. Effects of 2,4,6-Triethylenimino-*s*-triazine and Related Compounds on Transplanted Mouse Leukemia. Proc. Soc. Exper. Biol. & Med., **74**:708–12, 1950.
7. BURCHENAL, J. H.; LESTER, R. A.; RILEY, J. B.; and RHOADS, C. P. Studies on the Chemotherapy of Leukemia. I. Effect of Certain Nitrogen Mustards and Carbamates on Transmitted Mouse Leukemia. Cancer, **1**:399–412 1948.
8. FLORY, C. M.; FURTH, J.; SAXTON, J. A., JR.; and REINER, L. Chemotherapeutic Studies on Transmitted Mouse Leukemia. Cancer Research, **3**:729–43, 1943.
9. FERGUSON, F. C., JR.; THIERSCH, J. B.; and PHILIPS, F. S. Action of 4-Amino-N^{10}-methyl-pteroylglutamic Acid in Mice, Rats, and Dogs. Fed. Proc., **8**:291, 1949.
10. HITCHINGS, G. H.; ELION, G. B.; VANDERWERFF, H.; and FALCO, E. A. Pyrimidine Derivatives as Antagonists of Pteroylglutamic Acid. J. Biol. Chem., **174**:765–66, 1948.
11. KARNOFSKY, D. A.; BURCHENAL, J. H.; ORMSLEE, R. A.; CORNMAN, I.; and RHOADS, C. P. Experimental Observations on the Use of Nitrogen-Mustards in the Treatment of Neoplastic Disease. In Approaches to Tumor Chemotherapy. A.A.A.S., pp. 293–305, 1947.
12. LAW, L. W.; DUNN, T. B.; BOYLE, P. J.; and MILLER, J. H. Observations on the Effect of a Folic-Acid Antagonist on Transplantable Lymphoid Leukemias in Mice. J. Nat. Cancer Inst., **10**:179–92, 1949.
13. PHILIPS, F. S., and THIERSCH, J. B. Studies of the Actions of 4-Amino-pteroylglutamic acid in Rats and Mice. J. Pharm. & Exper. Therap., **95**:303–11, 1949.
14. SKIPPER, H. E.; EDWARDS, P. C.; BRYAN, C. E.; CHAPMAN, J. B.; BELL, M.; and HUTCHISON, O. S. Studies on the Antileukemic Action of Certain Compounds Related to Moieties of the 4-Amino-pteroylglutamic Acid (Aminopterin) Molecule. Cancer, **3**:348–53, 1950.
15. SKIPPER, H. E.; BENNETT, L. K., JR.; EDWARDS, P. C.; BRYAN, C. E.; HUTCHISON, O. S.; CHAPMAN, J. B.; and BELL, M. Anti-leukemic Assays on Certain Pyrimidines, Purines, Benzimidazoles, and Related Compounds. Cancer Research, **10**:166–69, 1950.
16. SUGIURA, K. The Effect of Roentgen Rays on the Growth of Mouse Sarcoma 180 Irradiated *in Vivo*. Radiology, **28**:162–71, 1937.
17. ———. Studies on Radiosensitivity of Mouse Sarcoma 180 Irradiated *in Vivo* and *in Vitro*. *Ibid.*, **29**:352–61, 1937.
18. SUGIURA, K., and COHEN, I. Further Study on the Direct and Indirect Actions of Radiation on Malignant Cells. I. Transplantation of Tissue Cultures of Mouse Sarcoma 180 after Exposure to Roentgen Rays. II. Cultures of Tumors Irradiated *in Vivo*. Radiology, **32**:71–76, 1939.

Inhibition by Testosterone of Radiation-induced Lymphoid Tumor Development in Intact and Castrate Adult Male Mice*

HENRY S. KAPLAN AND MARY B. BROWN

(*Department of Radiology, Stanford University School of Medicine, San Francisco, Calif.*)

Immature C57 black mice of both sexes develop lymphoid tumors with equal frequency after irradiation, and their susceptibility is not modified by gonadectomy (5). Several years ago, however, Murphy (12) noted that exogenous testosterone partially inhibited the development of spontaneous leukemias in Rockefeller Institute female mice, and Gardner (2) recently reported a reduced incidence of lymphoid tumors in irradiated female BC mice after testosterone administration. A pronounced degree of inhibition has been observed in post-pubertal male mice under the conditions of the experiment described herein.

METHODS

Litter-mate male C57 black mice were distributed among four groups at the time of weaning. Groups II and IV were castrated at 2 months of age, by a single-stage lower abdominal operation; the other two groups remained intact. Whole-body irradiation of all groups was started 3–4 days later, and a total dose of 673 r was delivered in four equally fractionated treatments at intervals of 4 days.[1]

Immediately after each irradiation, and continuing twice weekly for a total of 10 weeks, animals of groups III and IV were injected intramuscularly with 0.02 cc. of an aqueous suspension containing 0.5 mg. testosterone.[2] Groups I and II were similarly injected with an equal volume of the suspension medium alone.

All animals were identically caged and maintained on Purina Laboratory Chow and water ad libitum. Mice that died of any cause before the time of appearance of the first lymphoid tumor were omitted. Obviously moribund animals were killed, and all animals were carefully examined at autopsy; representative tissues were taken for histologic examination in all instances except where the presence of a mediastinal or disseminated lymphoma was obvious on gross inspection.

TABLE 1

EFFECT OF TESTOSTERONE ON THE DEVELOPMENT OF RADIATION-INDUCED LYMPHOMAS IN ADULT MALE C57 BLACK MICE

	Group	Material injected	Net no. of mice	Mice with lymphomas No.	Mice with lymphomas Per cent	Average latent period (days)	No. dead (neg.)	No. alive
I	Intact irradiated	Placebo	52	39	75	166	2	11
II	Castrate irradiated	Placebo	52	45	87	162	3	4
III	Intact irradiated	Testosterone	49	4	8	203	5	40
IV	Castrate irradiated	Testosterone	50	5	10	204	2	43

RESULTS

The data are summarized in Table 1, and cumulative incidence curves are presented in Chart 1. A striking inhibition of lymphoid tumor development resulted from the administration of testosterone to both intact and castrate adult male mice. Many animals of groups III and IV are still

* This investigation was supported by a grant from the National Cancer Institute, Public Health Service.

[1] Physical factors were: 120 kvP; 9 ma.; 0.25 mm. Cu and 1.0 mm. Al added filter; HVL = 0.36 mm. Cu; target-mouse distance, 30 inches; output, 32.2 r/min.

Received for publication April 28, 1951.

[2] Aqueous suspensions of testosterone, 25 mg/cc, stabilized with 0.35 per cent aluminum phosphate, and placebo vials of 0.35 per cent aluminum phosphate suspension were generously supplied by Dr. Edward Henderson of the Schering Corp., Bloomfield, N.J., with the information that such suspensions are somewhat more potent, in experiments of short duration, than testosterone propionate in oil. No studies relative to optimal dosage levels have as yet been made in our laboratory.

alive, but virtually no additional lymphoid tumors have appeared recently after an observation period of about 1 year, indicating that actual suppression or prevention, rather than mere delay in onset, has been effected. The slightly higher incidence among the castrate groups did not differ significantly from that of their respective intact controls. All the tumors were lymphocytic or lymphoblastic lymphomas, identical in histologic appearance and invasive behavior with those described previously (6).

An incidental observation during the period of injection was a considerable loss of hair, particularly over the ventral surface of the body, in the testosterone-treated animals. No similar alopecia was noted in the placebo-injected controls. There was a gradual return of hair after the series of testosterone injections was completed.

DISCUSSION

In experiments of short duration both testosterone and irradiation cause involution of lymphoid tissues (1, 13, 14). Information is sorely needed on the possible synergistic effect of the simultaneous administration of both agents on lymphoid tissues, and on the effect of testosterone on recovery of lymphoid tissues from radiation injury. Lacking such data, it seems premature to speculate at length upon the possible mechanism by which testosterone exerts such a striking inhibitory influence on the induction of lymphoid tumors.

The various steroid hormones that have been thus far studied do not exert a generically similar influence upon lymphoid tumor development. For example, estrogens induce lymphoid tumors in susceptible strains of mice (4, 9) and may synergize with x-radiation in their induction, whereas testosterone has not been independently leukemogenic (3) and tends to suppress leukemogenesis following irradiation. More recently, it was found that cortisone inhibits the development of lymphomas in irradiated mice, while desoxycorticosterone has no effect (7). The nature of the interaction between these hormones and lymphoid tissue is not sufficiently well understood at this time to permit an explanation of these diverse results.

Orchidectomy has been followed by an increase in spontaneous lymphoma incidence under certain experimental conditions (10–12). A similar result was not observed in the present investigation, possibly because the interval between castration and the first x-ray exposure was too short. Detailed studies of the optimal time intervals for castration and irradiation, and for irradiation and testosterone administration, are clearly indicated. It is of interest that the inhibitory effect of cortisone was still manifest when its administration was deferred until 6 weeks after irradiation (7). Parallel studies with testosterone are in progress.

SUMMARY

Intact and castrate male C57 black mice were irradiated and injected twice weekly for 10 weeks with an aqueous testosterone suspension; the corresponding litter-mate control groups were also irradiated and injected with a placebo of the suspension medium alone. Net lymphoid tumor incidence by groups was: (*a*) intact + x-ray + placebo, 39 of 52 mice (75 per cent); (*b*) castrate + x-ray + placebo, 45 of 52 mice (87 per cent); (*c*) intact + x-ray + testosterone, 4 of 49 mice (8 per cent); and (*d*) castrate + x-ray + testosterone, 5 of 50 mice (10 per cent). Some possible mechanisms for this pronounced inhibition are briefly discussed.

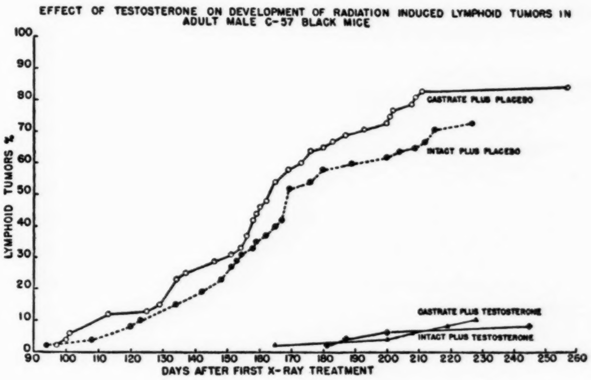


CHART 1

REFERENCES

1. BRECHER, G.; ENDICOTT, K. M.; GUMP, H.; and BRAWNER, H. P. Effects of X-Ray on Lymphoid and Hemopoietic Tissues of Albino Mice. Blood, **3:**1259–74, 1948.
2. GARDNER, W. U. Ovarian and Lymphoid Tumors in Female Mice Subsequent to Roentgen-Ray Irradiation and Hormone Treatment. Proc. Soc. Exper. Biol. & Med., **75:**434–36, 1950.
3. GARDNER, W. U.; DOUGHERTY, T. F.; and WILLIAMS, W. L. Lymphoid Tumors in Mice Receiving Steroid Hormones. Cancer Research, **4:**73–87, 1944.
4. GARDNER, W. U.; KIRSCHBAUM, A.; and STRONG, L. C. Lymphoid Tumors in Mice Receiving Estrogens. Arch. Path., **29:**1–7, 1940.
5. KAPLAN, H. S. Influence of Thymectomy, Splenectomy, and Gonadectomy on Incidence of Radiation-induced Lymphoid Tumors in Strain C57 Black Mice. J. Nat. Cancer Inst., **11:**83–90, 1950.
6. ———. Observations on Radiation-induced Lymphoid Tumors of Mice. Cancer Research, **7:**141–47, 1947.
7. KAPLAN, H. S.; BROWN, M.B.; and MARDER, S. N. Adrenal Cortical Function and Lymphoid Tumor Inci-

dence in Irradiated Mice. Cancer Research, **11**:262–63, 1951.

8. Kirschbaum, A.; Shapiro, J. R.; and Mixer, H. W. Synergistic Action of Estrogenic Hormone and X-Rays in Inducing Thymic Lymphosarcoma of Mice. Proc. Soc. Exper. Biol. & Med., **72**:632–34, 1949.
9. Lacassagne, A. Sarcomes lymphoïdes apparus chez des souris longuement traitées par des hormones oestrogènes. Compt. rend. Soc. de Biol., **126**:193–95, 1937.
10. Law, L. W. Effect of Gonadectomy and Adrenalectomy on Appearance and Incidence of Spontaneous Lymphoid Leukemia in C58 Mice. J. Nat. Cancer Inst., **8**:157–59, 1947.
11. McEndy, D. P.; Boon, M. C.; and Furth, J. On the Role of Thymus, Spleen, and Gonads in the Development of Leukemia in a High-Leukemia Stock of Mice. Cancer Research, **4**:377–83, 1944.
12. Murphy, J. B. Effect of Castration, Theelin, and Testosterone on Incidence of Leukemia in a Rockefeller Institute Strain of Mice. Cancer Research, **4**:622–24, 1944.
13. Plagge, J. C. The Thymus Gland in Relation to Sex Hormones and Reproductive Processes in the Albino Rat. J. Morphol., **68**:519–45, 1941.
14. Reinhardt, W. O., and Wainman, P. Effect of Thyroidectomy, Castration, and Replacement Therapy on Thymus of Male Rats. Proc. Soc. Exper. Biol. & Med., **49**:257–60, 1942.

Histochemical Demonstration of Esterase in Malignant Tumors*

RICHARD B. COHEN,† MARVIN M. NACHLAS,† AND ARNOLD M. SELIGMAN

(Yamins Laboratory for Surgical Research, Beth Israel Hospital, and the Department of Surgery, Harvard Medical School, Boston, Mass.)

A method for demonstrating nonspecific esterase in acetone-fixed and paraffin-imbedded tissue has been described by Nachlas and Seligman (9). β-Naphthyl acetate was used as a substrate, and sites of enzymatic activity were labeled by the production of an azo dye as in the methods for the phosphatases (7, 8, 13) and serum cholinesterase (12). About 60 per cent of the esterase activity of rat liver was destroyed by the fixation and imbedding procedures (9). Although acetylcholinesterase of fresh rat brain and serum cholinesterase are capable of hydrolyzing this substrate (12), acetone-fixation was considerably more destructive of the cholinesterase than ali-esterase. Lipase, which is also able to hydrolyze naphthyl acetate, was demonstrated in significant amount only in pancreatic homogenates (11, 14). Therefore, the procedure used in this study demonstrates almost exclusively nonspecific esterase activity.

A study of the nonspecific esterase activity of a series of malignant tumors forms the basis of this report.

METHOD

Tumor tissue[1] and normal tissue were obtained fresh at operation or at autopsy when the specimen could be removed within 4 hours of death. Tissue was fixed in cold acetone for 24 hours and imbedded in paraffin. Sections were cut 6 μ thick, stained for esterase, and mounted according to the procedure described previously (9). Photomicrographs[2] were taken through a green filter.

* This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

† Research Fellow of the National Cancer Institute, Federal Security Agency.

[1] Tumor tissue was provided through the courtesy of the Pathology Departments of the Beth Israel Hospital, New England Deaconess Hospital, and the Peter Bent Brigham Hospital, Boston.

[2] Photomicrography by Leo Goodman.

Received for publication April 30, 1950.

RESULTS

Results are given for 102 tumors according to the organ of origin (Table 1). The esterase activity of normal tissue is also given for comparison with the activity of the tumors. Esterase activity of tumor tissue was estimated by examination of three to seven slides of each tumor.

The nonspecific esterase content of most malignant tumors was low or entirely absent. In order to assess the role of fixation, fresh tissue was used in a few experiments. In three instances, tumors with low esterase activity had low esterase activity when fresh homogenates were used. In one instance, a tumor without esterase activity was devoid of esterase when a fresh homogenate was used. In no instance was esterase activity demonstrated in a tumor where the homologous normal tissue was devoid of such activity. These observations conform to the generalizations of Greenstein concerning the enzymatic pattern of normal and neoplastic tissue (4, 5).

Six of ten carcinomas of the stomach were devoid of esterase activity by this method. Four tumors contained slight activity (Fig. 1). Seventeen of 25 carcinomas of colon and rectum were devoid of esterase activity. Only one adenocarcinoma contained moderate activity (Fig. 2). Two carcinomas of the pancreas showed slight esterase activity in contrast to the intense enzymatic activity of pancreatic tissue. Only one of seven renal-cell carcinomas showed slight esterase activity (Fig. 3), and an intraductal papillary carcinoma of breast showed slight esterase activity. Fifteen breast carcinomas were devoid of esterase activity according to this method.

The most striking esterase activity was observed in five thyroid carcinomas (Fig. 4). In contrast to this finding, the follicle cells of the human thyroid contained slight esterase activity.

None of the remaining carcinomas and sarcomas showed esterase activity.

Wherever esterase activity was demonstrated, dye was deposited in finely divided intracyto-

TABLE 1

Nonspecific Esterase Activity in Acetone-fixed Malignant Tumors

Organ	Tumor	No. of tumors examined	Esterase activity*	Esterase activity* of normal tissue (10)
Esophagus	Epidermoid carcinoma	4	0	None
Stomach	Adenocarcinoma	2	+	Present in varying concentrations particularly in parietal cells (++)
	Adenocarcinoma	2	0	
	Undifferentiated (colloid) carcinoma	1	++	
	Undifferentiated carcinoma	1	+	
	Undifferentiated carcinoma	4	0	
Small intestine (duodenum)	Papillary adenocarcinoma	2	0	Slight in Brunner's glands (+)
Colon and rectum	Adenocarcinoma	1	+++	Slight in mucosal glands (++)
	Adenocarcinoma	4	++	
	Adenocarcinoma	3	+	
	Adenocarcinoma	17	0	
Pancreas	Adenocarcinoma	1	++	Intense in ducts and acini (++++)
	Adenocarcinoma	1	+	
Kidney	Renal-cell carcinoma	1	+	Variable and slight in tubules
	Renal-cell carcinoma	6	0	
Bladder	Transitional-cell bladder carcinoma	3	0	None
Breast	Adenocarcinoma	3	0	Variable and slight in ducts
	Intraductal papillary carcinoma	1	+	
	Undifferentiated carcinoma	12	0	
Ovary	Papillary carcinoma	3	0	None
	Undifferentiated carcinoma	2	0	
Uterus	Adenocarcinoma endometrium	2	0	Little in edometrial glands
Cervix	Squamous-cell carcinoma	3	0	None
Thyroid	Papillary adenocarcinoma	3	+++	Variable and slight in follicular cells (+)
	Undifferentiated carcinoma	1	+++	
	Undifferentiated carcinoma	1	++	
Testis	Seminoma	1	0	None
Prostate	Adenocarcinoma	1	0	Variable in epithelium
	Undifferentiated carcinoma			
Lung (bronchus)	Squamous-cell carcinoma	3	0	Weak and irregular in bronchial epithelium
Skin	Squamous-cell carcinoma	2	0	None
Tongue	Squamous-cell carcinoma	1	0	None
Pharynx	Transitional-cell carcinoma (Schminke)	1	0	
Brain	Glioblastoma multiforme	1	0	None
	Astrocytoma	2	0	
Mesenchyme (miscellaneous)	Lymphosarcoma (large-cell type)	1	0	None in lymph nodes
	Lymphosarcoma (small-cell)	2	0	
	Reticulum-cell sarcoma	1	0	
	Retroperitoneal myxosarcoma	1	0	None
	Fibrosarcoma of bone	1	0	None

* 0 = no esterase activity.
+ = slight esterase activity in isolated portions of the tumor.
++ = slight esterase activity throughout the tumor.
+++ = moderate esterase activity diffusely or focally located.
++++ = strong esterase activity (normal liver and pancreas).

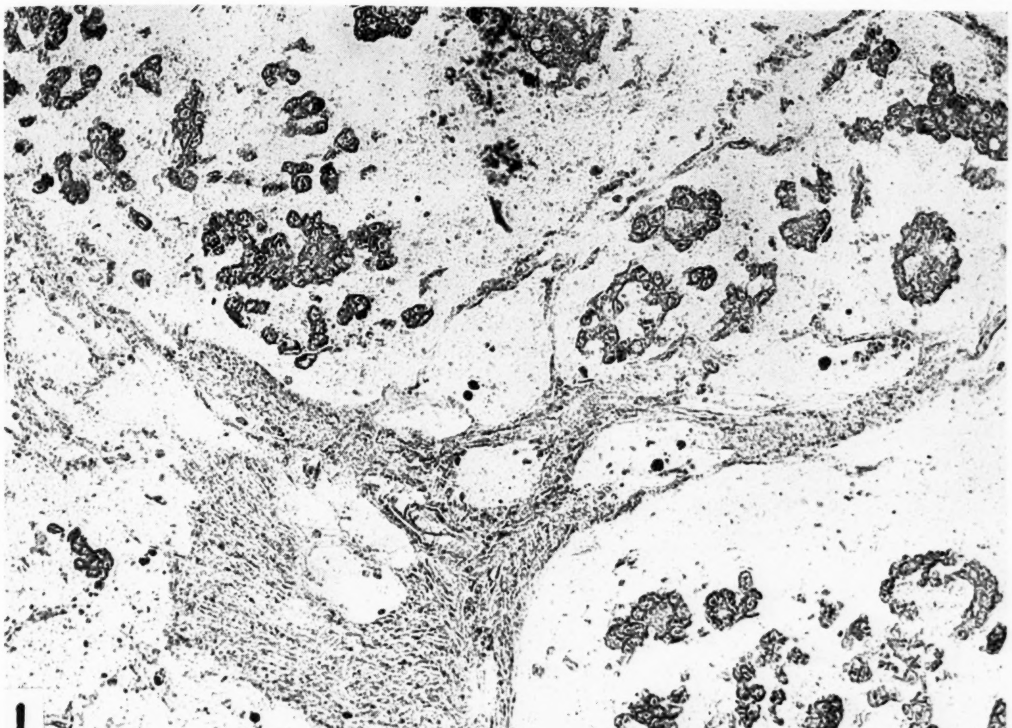

1
2
3

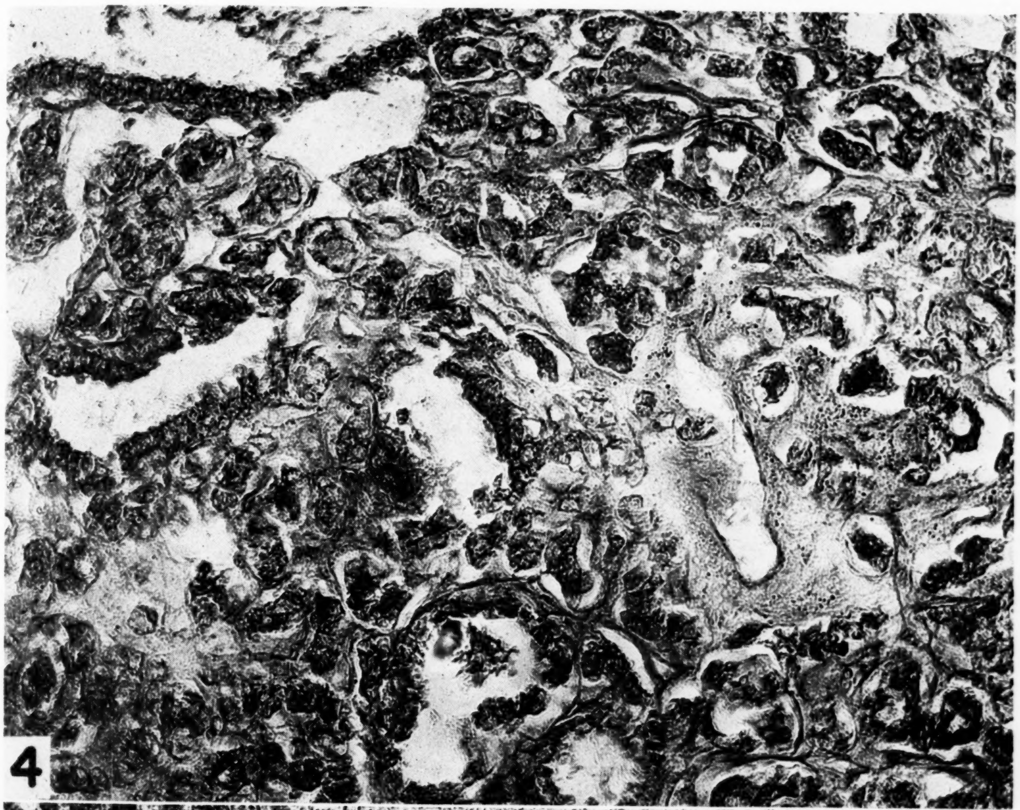
FIG. 1.—Colloid carcinoma of stomach. The cytoplasm of clusters of adherent tumor cells are uniformly stained for esterase. The surrounding colloid is unstained. ×400.

FIG. 2.—Adenocarcinoma of colon. Uniformly stained cytoplasm of adenocarcinoma contrasts with the unstained adjacent connective tissue (top). ×400.

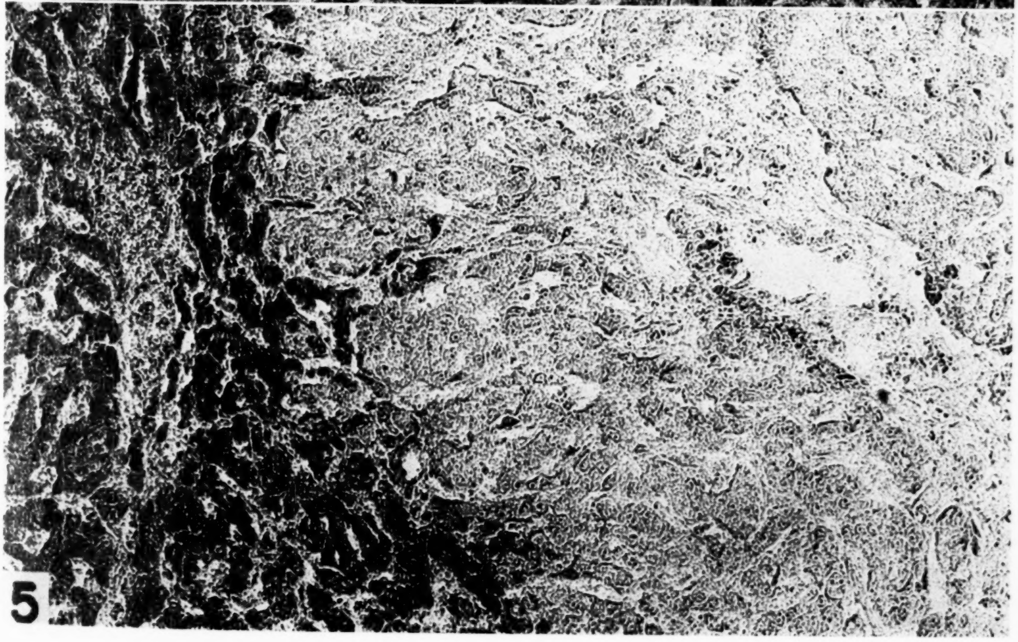
FIG. 3.—Renal-cell carcinoma. Fine granules of dye are present predominantly at the periphery of the clear tumor cells. Some of the cells are free of esterase activity, while other histologically similar cells have considerable activity. The nuclei are not stained. ×400.

FIG. 4.—Adenocarcinoma of thyroid. The cytoplasm of the tumor cells lining the acini is deeply stained. Some colloid material is weakly stained. ×400.

FIG. 5.—Undifferentiated carcinoma of breast metastatic to liver. Sharp contrast is apparent between intensely stained liver and the tumor which is devoid of esterase activity (right). ×400.



4



5

plasmic particles. Nuclear or stromal staining was not observed. No correlation between enzymatic activity and the degree of differentiation of the tumor was evident. Metastases of an esterase-free primary tumor were also free of esterase activity. Metastases of primary tumors which contained esterase activity usually contained enzymatic activity similar to that of the original growth. An intrahepatic metastasis from an esterase-free tumor stood out in bold relief in the surrounding normal, esterase-rich hepatic parenchyma (Fig. 5).

DISCUSSION

Esterases are ubiquitous in normal epithelial tissues of many species (10), particularly when fresh tissue is used. In the mouse, esterase activity toward methyl *n*-butyrate is greater in most normal tissues than in tumors (4). Most neoplasms are devoid of esterase activity toward Tween 40, 60, and 81 according to a histochemical technic (3).

It has also been shown that tumor tissue lowers the esterase activity of normal tissue. Thus, the esterase activity toward tributyrin of liver, brain, and blood in tumor-bearing animals is less than that in these tissues in normal animals (2). The same is true in rats with a transplanted adenocarcinoma, when ethyl butyrate is used as substrate (16). The blood esterase dropped after implantation of the tumor and rose again after removal of the tumor.

On the other hand, it has been reported (1, 6) that serum butyric esterase is about twice as high in two strains of cancer-susceptible mice (A and C3H) as in cancer-resistant strains (C57 black). This has been correlated with excretion of the enzyme rather than with its production in the liver.

The consistently low esterase activity of human tumors (except thyroid), compared to normal epithelial tissues and blood (14), may be useful in providing a rationale for the development of possible chemotherapeutic agents (15). Cytotoxic esters of mustards whose split products are considerably less toxic than the esters, might prove to be specifically destructive of tumors low in esterase activity. Such a study is in progress.

SUMMARY

1. The nonspecific esterase activity of most human tumors is low or frequently absent.

2. Carcinomas have less esterase activity than the homologous tissue of origin.

3. In contrast to other tumors, carcinomas of the thyroid have considerable esterase activity.

4. The esterase activity does not correlate with the degree of differentiation of the tumor.

REFERENCES

1. Chitre, R. J., and Khanolkar, V. R. Studies in Esterase (Butyric) Activity. II. Esterase Content of Serum of Mice and Its Excretion in Strains Susceptible or Unsusceptible to Mammary Cancer. Cancer Research, **3**:88–91, 1943.
2. Edlbacher, S., and Neber, M. Über das Lipasespaltungsvermögen der Säugerorgane im gesunden und tumorkranken Organismus. Ztschr. Physiol. Chem., **233**: 265–75, 1935.
3. Gomori, G. Distribution of Lipase in the Tissues under Normal and under Pathological Conditions. Arch. Path., **41**:121–29, 1946.
4. Greenstein, J. P. Esterase (Butyric Esterase) Activity of Normal and Neoplastic Tissues of the Mouse. J. Nat. Cancer Inst., **5**:31–34, 1944.
5. ———. Biochemistry of Cancer, p. 198. New York: Academic Press, 1947.
6. Khanolkar, V. R., and Chitre, R. J. Studies in Esterase (Butyric) Activity. I. Esterase Content of Serum of Mice from Certain Cancer-resistant and Cancer-susceptible Strains. Cancer Research, **2**:567–70, 1942.
7. Manheimer, L. H., and Seligman, A. M. Improvement in the Method for the Histochemical Demonstration of Alkaline Phosphatase and Its Use in a Study of Normal and Neoplastic Tissues. J. Nat. Cancer Inst., **9**:181–99, 1948.
8. Menten, M. L.; Jung, J.; and Green, M. H. A Coupling Histochemical Azo Dye Test for Alkaline Phosphatase in the Kidney. J. Biol. Chem., **153**:471–77, 1944.
9. Nachlas, M. M., and Seligman, A. M. Histochemical Demonstration of Esterase. J. Nat. Cancer Inst., **9**:415–25, 1949.
10. ———. The Comparative Distribution of Esterase in the Tissues of Five Mammals by a Histochemical Technique. Anat. Rec., **105**:677–96, 1949.
11. ———. Evidence for the Specificity of Esterase and Lipase by the Use of Three Chromogenic Substrates. J. Biol. Chem., **181**:343–55, 1949.
12. Ravin, H. A.; Tsou, K. C.; and Seligman, A. M. Colorimetric Estimation and Histochemical Demonstration of Serum Cholinesterase. J. Biol. Chem., **190**:391–402, 1951.
13. Seligman, A. M., and Manheimer, L. H. A New Method for the Histochemical Demonstration of Acid Phosphatase. J. Nat. Cancer Inst., **9**:427–34, 1949.
14. Seligman, A. M., and Nachlas, M. M. The Colorimetric Determination of Lipase and Esterase in Human Serum. J. Clin. Investigation, **29**:31–36, 1950.
15. Seligman, A. M.; Nachlas, M. M.; Manheimer, L. H.; Friedman, O. M.; and Wolf, G. Development of New Methods for the Histochemical Demonstration of Hydrolytic Intracellular Enzymes in a Program of Cancer Research. Ann. Surg., **130**:333–41, 1949.
16. Troescher, E. E., and Norris, E. R. A Micro Blood Esterase Determination Applied to Studies of Rats Bearing Adenocarcinoma. J. Biol. Chem., **132**:553–57, 1940.

The Estrous Behavior and Mammary Cancer Incidence in Ovariectomized C3H Mice in Relation to Calorie Intake*

JOSEPH T. KING, CARMEN B. CASAS,† AND MAURICE B. VISSCHER

(Department of Physiology, University of Minnesota, Minneapolis, Minn.)

The paradoxical occurrence of a continuous state of sub-estrus associated with adrenal cortical hyperplasia and metaplasia in ovariectomized mice of certain strains was first observed by Woolley, Fekete, and Little (3). The present authors (1) have noted that caloric restriction prevents the occurrence of estrus, without abolishing the cellular changes in the adrenal cortex in ovariectomized C3H mice. The study herein reported was undertaken in order to obtain data concerning (*a*) the time required for the state of continuous sub-estrus to be abolished when previously fully fed castrate mice are restricted in dietary caloric intake, (*b*) the time required for previously caloric-restricted castrate mice to go into continuous estrus when allowed food ad libitum, and (*c*) the mammary cancer incidence in the situations outlined in (*a*) and (*b*). The reversals in dietary situations were made at several ages in order to test the influence of age upon the capacity for functional alterations.

MATERIALS AND METHODS

One hundred C3H mice were ovariectomized at 21–23 days of age. A group of twenty was full-fed throughout the experiment; a group of ten was similarly restricted. Four groups of ten each were started on restriction and reversed to full feeding after approximately 2, 3, 6, and 8 months. Three groups of ten each were started on full feeding and reversed to restriction at approximately 2, 3, and 6 months. Vaginal smears, made by the lavage method, were started before diet reversal. Smears from the group full-fed throughout life were made only at intervals, and hence smear data are not included in the graph. In corroboration of earlier findings, it was found that these animals showed a continuous state of sub-estrus from about the third month of life. Smears were recorded as positive when dense and showing varying proportions of leukocytes, epithelial cells, and cornified cells. Smears were discontinued at 14 months. The mice were housed individually in an air-conditioned room at 78° ± 4° F. and were weighed weekly. The diet used was that described by Visscher, Ball, Barnes, and Sivertsen (2). The restricted animals received approximately the same amount of protein, minerals, and vitamins with amounts of fat and carbohydrate reduced to provide 66 per cent of the caloric intake of the controls.

RESULTS

The major findings are shown in Charts 1 and 2. In Chart 1 the time at which continuous sub-estrus began is not shown, but eighteen of twenty mice full-fed for more than 2 months went into estrus. The bars crossing the columns in this chart indicate the times of permanent disappearance of the estrous state. It can be noted that the estrogenic response in the animals was lost in all but three mice 4 months after institution of caloric restriction. The time intervals did not vary greatly in the groups reversed at 3 and 6 months. In the group restricted at 2 months only one mouse came into estrus, and this state persisted until the thirteenth month. This animal was clearly an exception and corresponds to the single mouse in Group IX which showed estrus on life-time restriction.

In Chart 2 the cross bars indicate the time of going into the estrous state. Here again it will be noted that in all age-at-reversal groups most of the mice showed continuous sub-estrus within 4 months after reversal to full feeding. There is an indication that the interval is shortest in Group VII, which was reversed at 6 months.

These points are brought out in Table 1, which also gives some of the body weight data. In particular it is to be noted that the mean body

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service.

† Present address: Department of Physiology, University of Puerto Rico, San Juan.

Received for publication May 1, 1951.

weights found when smears became positive for animals reversed from restriction to full feeding are greater in all instances than the mean body weights found when smears became negative in animals reversed from full feeding to caloric restriction.

The mammary tumor data are summarized in Table 2. It will be seen that only in two groups was the mammary tumor incidence less than 50 per cent. These were the mice restricted for their lifetimes and from 2 months of age, respectively. In all the other groups it is apparent that conditions were not unfavorable to mammary carcinogenesis. Except for the single tumor in the group restricted for a lifetime, the median tumor age followed the expected pattern for mice on full diets. The single animal in the lifetime restriction group showing mammary cancer at 10 months is obviously a random exception.

DISCUSSION

These results confirm and extend the earlier observations (1) which indicated that the function

TABLE 1

EFFECT OF CHANGES IN CALORIC INTAKE ON ESTRUS IN OVARIECTOMIZED C3H MICE

Restricted to ad libitum at (mo.)	Av. wt. at reversal (gm.)	Av. wt. gain at pos. smear (gm.)	Av. wt. at pos. smear (gm.)	Median time for pos. smear (mo.)
2	16.1	12.3	28.4	1.8
3	20.0	5.6	25.6	2.2
6	19.8	7.8	27.6	0.8
8	20.2	9.2	29.4	2.5
Ad libitum to restricted at (mo.)		Av. wt. loss at neg. smear (gm.)	Wt. at neg. smear (gm.)	Neg. smear after restriction (mo.)
6	36.3	12.8	23.5	2.0
3	29.9	8.9	21.0	3.3
2	24.2	*	*	*

* Did not go into estrus, except for one aberrant mouse.

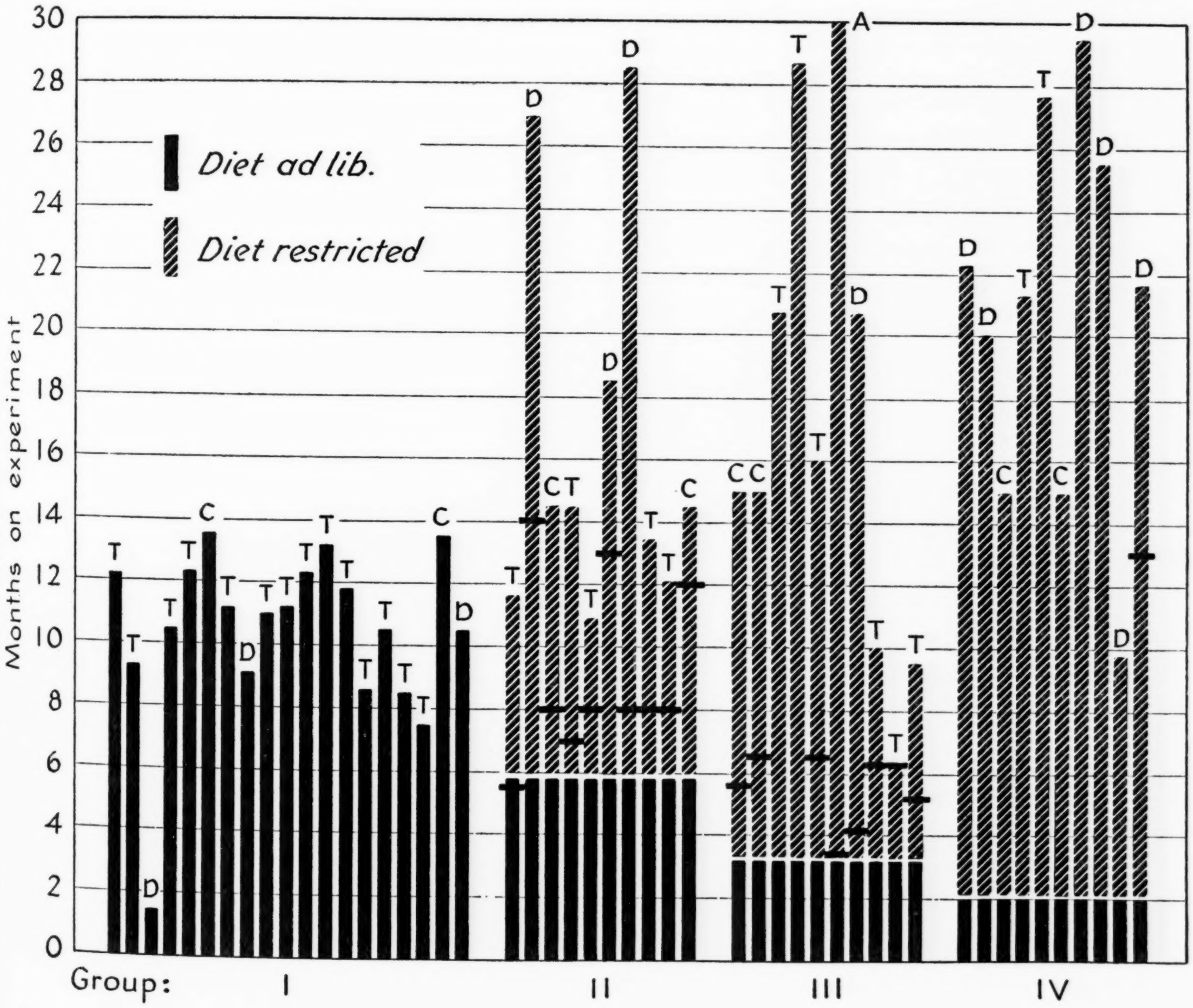


CHART 1.—Life span, estrous response, and mammary tumor incidence in ovariectomized C3H mice maintained on full feeding (Group I), on full feeding for varying periods in Groups II, III, and IV, and reversed to one-third caloric restriction at the time indicated. The horizontal bars across the vertical columns indicate the times of cessation of continuous sub-estrus. *T* indicates death due to mammary cancer; *D*, death due to other causes; *C*, sacrifice for other studies; *A*, died noncancerous at 33 months.

of the estrogen-producing structures in the castrate C3H mouse is critically determined by caloric intake. If, as it seems likely, the adrenal cortex is the structure involved, these results prove that either directly or indirectly the estrogen output of the adrenal cortex is controlled by caloric intake. There are as yet no data from which to conclude whether the effect is a direct one on the cortex or an indirect one mediated through the hypophysis. On *a priori* grounds either mechanism is possible, but, since ovarian estrogen output during caloric restriction is controlled indirectly by the pituitary, it is perhaps more likely that the same will be found in the case of the adrenal cortex.

TABLE 2

EFFECT OF CALORIC INTAKE ON THE MAMMARY TUMOR INCIDENCE IN OVARIECTOMIZED C3H MICE

Diet history	No. of mice*	No. with mammary tumors	Median tumor age (mo.)
Full-fed throughout	17	14	11
" 6 mo., then restricted	8	5	12
" 3 mo., " "	8	6	11
" 2 mo., " "	8	2	21
Restricted throughout	7	1	10
Restricted to 8 mo., then full-fed	8	6	14
" 6 mo., " "	7	4	14
" 3 mo., " "	8	4	11
" 2 mo., " "	7	6	11

* Excluding mice sacrificed for chemical study.

The primary purposes of this study were to ascertain whether the age at reversal in the feeding pattern crucially altered the character of the response. It is obvious that up to 8 months of age there is no loss in the capacity of the mouse to respond to changes in calorie intake. Upon reversal from restriction to full feeding at the later ages, the high ensuing mammary cancer incidence is undoubtedly caused in part by the prolonged life-

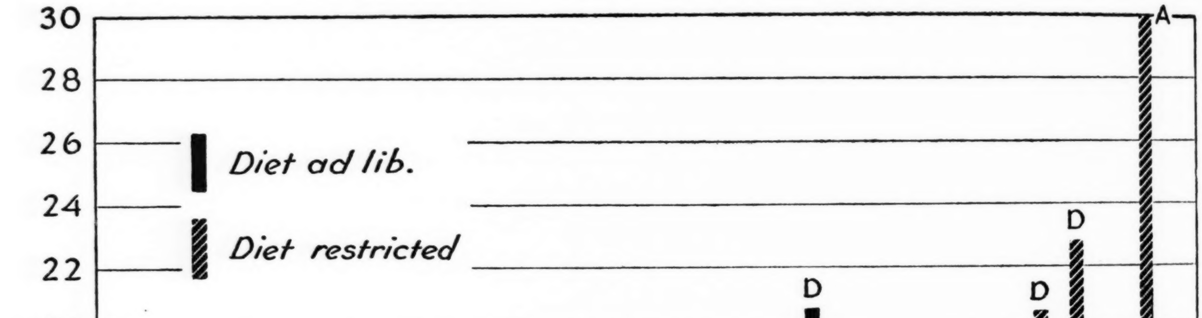


CHART 2.—Symbols as in Chart 1, except that the horizontal cross bars indicate the time of onset of positive estrus smears. In Groups V, VI, VII, and VIII, the diet reversals were from restriction to full feeding.

span due to caloric restriction.[1] Nevertheless, it is of interest that 8 months of caloric restriction, sufficient to hold body weight to 20.2 gm. (Table 1), while full-fed animals at 6 months had body weights averaging 36.3 gm., was unable to influence greatly the ultimate mammary cancer incidence. Any mouse which has free access to calories for an average period of 3 months or more at any time during its life span has a resulting high mammary cancer incidence under the conditions employed in this study. Thus, it appears that full feeding for about 25 per cent of the full-feeding life span is a minimum condition for a high rate of mammary carcinogenesis under the conditions described above. If one considers the life span in the C3H mouse lacking the mammary tumor milk agent, this proportion would, of course, be different, because the life span is greater. The fraction would fall to about 12 per cent of the average duration of life.[1]

CONCLUSIONS

In the ovariectomized C3H mouse, caloric underfeeding inhibits the occurrence of the continuous sub-estrus which is seen in full-fed mice. When previously full-fed mice are restricted in calories at various ages, the positive estrous smear disappears in most cases, in about 3 months. When calorie-restricted mice are reversed to full feeding, estrus is induced in from 1 to 4 months. The body weight changes associated with reversals in response are greater in the case of disappearance of estrogenic activity. The mammary tumor incidence in these mice is above 50 per cent in all groups which were full-fed for 3 months or more at some time during life. Therefore, it can be said that relatively short periods of full caloric feeding are sufficient to induce those developments essential to mammary carcinogenesis. Full feeding for 2 months or less is inadequate to permit a high cancer incidence in C3H ovariectomized female mice when such feeding immediately follows weaning, i.e., while the mice are relatively immature. The question whether the full feeding of restricted mice for a 2-month period later in life would produce the same result cannot be answered on the basis of these experiments.

[1] Author's unpublished data.

REFERENCES

1. KING, J. T.; CASAS, C. B.; and VISSCHER, M. B. Effect of Caloric Restriction on the Adrenal Response of Ovariectomized C3H Mice. Am. J. Physiol., **157**:193–96, 1949.
2. VISSCHER, M. B.; BALL, Z. B.; BARNES, R. H.; and SIVERTSEN, I. The Influence of Caloric Restriction upon the Incidence of Spontaneous Mammary Carcinoma in Mice. Surgery, **11**:48–55, 1942.
3. WOOLLEY, G.; FEKETE, E.; and LITTLE, C. C. Effect of Castration in the Dilute Brown Strain of Mice. Endocrinology, **28**:341–43, 1941.

The Heterologous Transplantation of Human Tumors*

ABE TOWBIN

(*Department of Pathology, Ohio State University, Columbus, Ohio*)

INTRODUCTION

The transplantability of human tumors to the guinea pig eye has been proposed as the basis for a test to differentiate benign from malignant growths. Although in most instances the character of a neoplasm can be accurately defined by histological study, there remains an important number of cases in which the microscopic diagnosis is equivocal; here it would be of great value to have a feasible animal test which could be used to augment the histopathological analysis of the tumor.

The study of tissue malignancy by intra-ocular transplantation has been the center of considerable interest in recent years. Although this method has been used in some laboratories as a diagnostic measure with apparent success (1, 2, 4), some investigators have expressed doubt as to the theory and the clinical applicability of the procedure (3, 5). To ascertain the value of this method as a diagnostic measure, it must be demonstrated that a large percentage of clinically malignant human tumors grow when transplanted to the guinea pig eye; if, in addition to benign growths, most malignant tumors prove to be nontransplantable, the diagnostic value of this procedure would be limited. Clarification of this matter awaits the accumulation of independent statistical studies in which this technic has been used. Greene and Lund (2), Newton (4), and others have recommended the use of the anterior chamber technic in the differentiation of benign and malignant tumors. On the other hand, Schilling, Snell, and Favata (5) observed transplant growth with only 6 of 36 human malignancies studied by this method; results in this series were interpreted on the basis of gross enlargement or histologic evidence of growth. Morris, McDonald, and Mann (3) studied 40 different human tumors, using 170 ocular transplants; in this series no evidence of tumor growth was noted. An evaluation of the anterior chamber technic as a diagnostic measure has been carried on in this laboratory during the last 2 years; 100 different human malignant tumors and 38 malignant tumors of lower animals have been studied; 1,670 tissue transplants have been analyzed.

Theory.—The use of this procedure as a diagnostic measure is based upon the premise that there is a consistent relationship between the malignancy and the transplantability of a tumor. A comprehensive résumé of the literature dealing with the theoretical aspects of this problem has been recently presented by Morris, McDonald, and Mann (3). Clinically, the growth momentum of a tumor, i.e., the rate of enlargement, infiltration, and metastasis, characterizes the degree of malignancy of a neoplasm. It has been shown experimentally with animal tumors that growth momentum is likewise one of the most important factors governing transplantability, particularly heterologous transplantability. Tumors of low growth momentum are transplantable only to members of the same or closely related strains of animals. However, tumors with rapid growth momentum have been transplanted successfully to other strains and species. Accordingly, the determination of heterologous transplantability of a tumor would provide a measure of its growth momentum and, hence, the degree of malignancy.

Working with spontaneous tumors of rabbits, Greene found that heterologous transplantation failed during the early stages of neoplasia, when growth momentum was manifestly low. At a later point in the development of the tumor, when growth momentum had increased and there was evidence of infiltration and metastasis in the original host, heterologous transfer of the tumor became uniformly successful. Likewise, theoretically, a basic growth momentum consistent with clinical malignancy must be attained by a human neoplasm before it can be transplanted successfully to the guinea pig. The tenability of this theory depends upon the results of studies comparing the growth of transplanted tumor in the guinea pig eye with the behavior of the same neoplasm clinically in the patient.

In the transplantation of tumor tissue from one species to another, the receptivity of the tissue of

* This project was supported by grants from the National Cancer Institute of the National Institutes of Health, Public Health Service.

Received for publication May 8, 1951.

the new host is an important factor. The tissue of the transplanted animal, in varying degrees, offers resistance to the growth of the foreign tumor. This resistance is, in general, governed by the degree of species difference between the donor animal and the new host. For example, the tissue of the guinea pig eye offers relatively little resistance to growth when it receives malignant tumors of mice or other closely related species. With comparable tumors of dog, horse, and other higher animals, growth occurs less readily because of the increased species barrier. If the method proposed by Greene is to have practical application, it must be demonstrated that clinically malignant human tumors are able to overcome the species barrier involved in transplantation to the guinea pig. The experimental examination of this issue forms the basic problem of the present investigation.

Principles of evaluation.—Comparison of reports of investigators studying this procedure reveals considerable divergence in results and conclusions. To a large extent this disagreement registered in the literature is the result of faulty application of precise experimental standards.

First, before definitive studies are initiated, proficiency with the transplantation technic and standardization of the laboratory routine must be achieved. Second, during the evaluatory studies with the human tumors a simultaneous control series with the use of known transplantable animal tumors must be maintained so that effects of external factors may be recognized. Third, results of each transplantation experiment must be based upon accurately correlated histologic studies of tissue from the original tumor and tissue from the transplanted site. Fourth, the study must be statistically valid; the series must be of appropriate proportions; the report must clearly indicate not only the number of successful, but also the unsuccessful, procedures carried out during the investigation.

A comprehensive conclusion in this problem, as with other current procedures for cancer diagnosis, can be evolved only by the accumulation of studies in which proper experimental precepts have been scrupulously maintained.

MATERIALS AND METHODS

Tissue was transplanted from 100 malignant human tumors obtained from surgical specimens and from fresh autopsy material in the Department of Pathology at Ohio State University. Tissue from 38 animal tumors was provided by the College of Veterinary Medicine at Ohio State University. In the tumors used, the diagnosis of malignancy was based upon the growth and behavior of the neoplasm in the patient. In most instances metastases to lymph nodes were demonstrated at operation. In some cases the malignant nature of the tumor was indicated by criteria of rapid growth, infiltration, or recurrence at the primary site, or by x-ray evidence of metastasis. Of the human tumors studied, 96 specimens were obtained at surgery; in four cases in which necropsy was performed directly after death, autopsy material was used (Table 2). In the study of animal tumors, tissue was obtained immediately after the animals were sacrificed.

A portion of each specimen was studied histologically. Cellular, well preserved tumor tissue was selected. Immediately after the specimen was obtained, the tumor tissue was transplanted into the anterior chamber of the eyes of eight guinea pigs; one eye was used in each animal, and equal numbers of males and females were used in each series. The animals were lightly anesthetized with Nembutal, administered intraperitoneally; in addition, a local anesthetic was applied to the cornea. Under sterile conditions the tissue was introduced into the anterior chamber with a No. 16 trocar through a small incision in the upper sclerocorneal junction. The tissue fragment was maneuvered into the lower angle of the chamber by stroking the outer surface of the cornea with a blunt probe.

Greene (1) has indicated that heterologous transplantation of human tumors is more often successful with the guinea pig than with any other laboratory animal. The anterior chamber has been selected as the site of transplantation, because foreign body and immune reaction appears to be less here than in other tissues. Constant observation of the transplant is possible through the transparent cornea.

Tissue was harvested from the guinea pig when the tumor filled the anterior chamber, or sooner if the onset of degenerative changes such as blanching or discoloration became apparent. Final evaluation was based upon the optimal result obtained in the series of anterior chamber transplantations performed with each tumor.

Standardization and control experiments were done during the preliminary phase of the study in order to establish criteria for interpreting results. In formulating these standards, use was made of mouse tumors, particularly the C3H mammary carcinoma and the Sarcoma 37; these tumors were useful because they grew well in the anterior chamber of the guinea pig, and a uniform supply of standard tissue was available for comparison studies. In the period during which the human tumors were evaluated, a simultaneous control

series with mouse tumors of known transplantability was constantly maintained so that the effect of external factors might be recognized. In addition, tumors from a variety of other lower animals were transplanted to the guinea pig in order to observe the relationship between degree of species difference and tumor transplantability.

Method of Interpreting Results

The lack of a comprehensive method of interpreting results has been one of the major pitfalls in this field of investigation. In studies with human tumors, conclusions based upon *macroscopic* observation of transplant growth in the anterior chamber are not reliable. The gross differentiation between early growth of a transplant and a foreign body inflammatory reaction is often mere speculation. Often the grossly observed initial transplant enlargement, even up to 3 times the original size, proved on histological examination to be due entirely to an inflammatory reaction. Final interpretation of results must be substantiated by histological studies of tissue harvested from the anterior chamber.

The terms "take," "positive," and "negative" will not be used in recording the results in this study because of the latitude which these terms have acquired in this field of investigation.

Studies indicate that tumor transplants in the anterior chamber evolve through three well defined stages: Suspension—Nidation—Growth. This pattern of transplant development provides a basis for the interpretation of results in studies in which the anterior chamber technic is used (6).

Suspension stage.—During the first phase, which usually lasts from 3 to 6 days, the tumor fragment exists in a "tissue culture" state. Essentially, this stage provides for the temporary subsistence of the new transplant. The tumor fragment lies loosely suspended between the cornea and iris in the lower portion of the anterior chamber (Figs. 1 and 2). The integrity of the surface cells of the transplant is maintained by the aqueous humor. The core of the transplant undergoes necrosis; if this degeneration extends to the periphery, the entire transplant regresses. Although some overlapping occurs between the suspension stage and that of nidation and growth, the series of changes described are, generally, well delineated.

Nidation stage.—The principal effect of the second stage is to bring into close association vascular elements of the host with tumor cells of the transplant. The tumor fragment becomes organized and attached to the iris. Nutrition of the tumor tissue by the aqueous humor is replaced by direct support of the transplant by the vascular system of the new host. Observed through the cornea the transplant becomes pink and vascularized but usually shows little or no enlargement. Histologically, the transplanted fragment, attached to the iris, contains vascular elements which arise from the iris of the guinea pig (Fig. 3). The period of nidation is usually short, varying from 3 to 7 days, and in successful transplantations is followed by the final phase—active growth. In many instances, a fixed state of nidation may endure for many months; tumor cells of the transplant are maintained in a viable form, but proliferation is not evident. This long latent period may eventually proceed to active growth or may terminate in regression of the transplant.

Growth stage.—The sustained growth which characterizes this stage is due to multiplication of tumor cells from the transplant accompanied by increased proliferation of vessels and other stromal elements from the host. With tumor tissue of rapid growth momentum, such as mammary carcinoma of the C3H mouse, pseudopodia-like extensions from the margin of the pink vascularized transplant may be observed grossly as early as the sixth day; the anterior chamber may become filled by the 21st day; extension beyond this limit was not observed in this study. Regression of the tumor tissue may follow an initial period of strong growth. Differentiation of tumor tissue is sometimes evident in heterologously grown tumor tissue; mammary carcinoma of the C3H mouse in its original state is a cellular tumor with few small acini; when grown in the guinea pig this tissue consistently shows dilated glandular spaces filled with clear pink-staining material (Fig. 4).

The three-stage pattern of development demonstrated by transplants in the anterior chamber furnishes a system for evaluating results. Tumors may be classified according to the maximal stage of development attained by transplants in the guinea pig eye. Accordingly, results are conveniently divided into the following three groups:

Transplants showing growth (*Group I*).—Included in this category are the tumors which on transplantation progressed through the suspension and nidation stages and showed frank growth. Tissue harvested 10 days or more after transplantation showed a histologic pattern similar to the original tumor.

Transplants persisting in the stage of nidation (*Group II*).—Included in this category are the tumors which on transplantation likewise evolved through the suspension stage and established nidation. Firm attachment to the iris and vascularization were evident, but significant enlargement

did not occur. Histologically, the tissue from the anterior chamber showed well preserved vascularized tumor elements. The integrity of the transplanted tissue was maintained in this manner for periods from 10 to 144 days.

Transplants showing early regression (*Group III*).—Included in this category are tumors which on transplantation failed to establish definite nidation and growth. Most of the transplants in this group disintegrated during the suspension stage. A few showed evanescent vascularization but on histologic examination showed only an inflammatory reaction. In some cases nidation was prevented by post-transplantation ocular infections.

RESULTS

The results of transplantation experiments with 100 malignant human tumors and 38 malignant tumors of lower animals are summarized in Table 1.

HUMAN TUMORS

The human tumors transplanted in this study are listed in Table 2. Tumors are classified as Groups I, II, and III, according to the maximal stage of development attained by transplants in the guinea pig eye.

Group I (*transplants reaching the stage of growth*). —Of 100 human tumors studied, transplants of two showed strong growth.

1. *Fibrosarcoma*, metastatic to the lung (tumor no. 6Y): The primary tumor in the arm had been resected 5 years previously. A late metastasis had appeared in the lung; tissue for transplantation was obtained from this specimen. Microscopically, the tumor was composed of irregular bundles of elongated fibrocytic elements showing numerous mitoses. Of the eight transplants, one showed evidence of growth at 74 days. The anterior chamber became filled with soft, gray, friable tissue at 175 days. The microscopic picture of the harvested tissue closely resembled the original tumor (Figs. 5 and 6).
2. *Carcinoma, maxillary sinus* (tumor no. 9T): tissue was transplanted from the deep portion of the primary site. Clinically, this tumor was an infiltrating carcinoma of the maxillary sinus. Histologically, it was a well differentiated cellular adenocarcinoma. Of the eight transplants, one showed evidence of growth on the 66th day. At the 117th day, the tumor filled one-third of the anterior chamber. Harvested tissue showed an adenocarcinoma pattern similar to the original tumor (Figs. 7 and 8).

Group II (*transplants persisting in the stage of nidation*).—Transplants of nine human tumors comprising this group showed successful nidation with gross vascularization but no significant enlargement; harvested tissue showed preserved tumor elements histologically.

1. *Carcinoma of the colon* (tumor no. 1N): one of the eight transplants persisted and was harvested on the 114th day.
2. *Carcinoma of the adrenal cortex* (tumor no. 3G): two of the eight transplants persisted; one transplant harvested on the twentieth day showed preserved tumor elements; the remaining similar transplant slowly regressed.
3. *Malignant melanoma* (tumor no. 3P): transplant harvested on tenth day showed well vascularized tumor nodule attached to the iris.
4. *Carcinoma of ovary* (tumor no. 5I): four of the transplants showed evidence of nidation; two showed well preserved tumor elements when harvested at 12 days; the remaining two were grossly vascularized but later regressed.

TABLE 1

THE RESULTS OF HETEROLOGOUS TRANSPLANTATION OF 100 HUMAN AND 38 ANIMAL TUMORS

	Human	Animal
Group I. Transplants of this group of tumors attained the stage of growth	2	6
Group II. Transplants of this group of tumors persisted in the stage of nidation	9	5
Group III. Transplants of this group regressed during the stage of suspension	89	27
Total	100	38

5. *Sarcoma, neurogenic* (tumor no. 7B): one of eight transplants persisted. Tissue harvested on the fourteenth day showed a sarcomatous pattern similar to the original tumor.
6. *Carcinoma of thyroid* (tumor no. 8C): the animal died on the tenth day. Tissue recovered from the anterior chamber showed well preserved carcinoma elements imbedded in the iris of the guinea pig eye.
7. *Carcinoma of the bronchus* (tumor no. 8S): the marginal layer of the nodule recovered from the anterior chamber on the 55th day showed elements similar to that of the undifferentiated carcinoma pattern of the original tumor.
8. *Sarcoma, neurogenic* (tumor no. 9D): a tumor nodule recovered from the anterior chamber of the guinea pig on the 26th day closely resembled the well differentiated original tumor pattern.
9. *Carcinoma of the bronchus* (tumor no. 10A): a nodule of tumor tissue recovered on the 21st day showed nests of epithelial tumor cells similar to that of the original tumor.

Group III (*tumors undergoing early regression*). —Of the 100 human tumors transplanted, 89 un-

derwent early regression; of this group 61 degenerated while in the suspension stage. Twelve tumors included in Group III showed faint vascularization of transplants; subsequently, blanching occurred, and histologic examination of the harvested transplant showed only degenerating elements and fibrosis. Although some of these tumors theoretically might have been classified in Group II, this was not done, because histologic verification was not available.

Transplantation results with sixteen of the tumors in Group III were complicated by severe ophthalmitis which was thought to be caused largely by the use of infected tumors; this occurred despite precaution to exclude such specimens. Since this procedure has been proposed for regular laboratory use and since the factor of bacterial infection of tumor tissue often cannot be predicted, these tumors should be included in the final evaluation of the method.

Details of tumors included in Group III are listed in Table 2.

TABLE 2

HUMAN TUMORS TRANSPLANTED TO THE ANTERIOR CHAMBER OF THE EYE OF GUINEA PIGS

Group	Type of tumor	Source of tissue: Primary site	Lymph node	Metastatic site: Lung	Bone	Liver	Total no.
I	Carcinoma, maxillary sinus	1					1
	Fibrosarcoma, arm			1			1
II	Carcinoma, colon		1				1
	Carcinoma, adrenal cortex	1					1
	Carcinoma, ovary	1					1
	Carcinoma, thyroid		1				1
	Carcinoma, bronchus		2				2
	Sarcoma, neurogenic	2					2
	Malignant melanoma, skin		1				1
III	Carcinoma, larynx		3				3
	Carcinoma, kidney	2		1			3
	Carcinoma, ovary	1	2*				3
	Carcinoma, anus		1				1
	Carcinoma, bladder		3				3
	Carcinoma, stomach		4*				4
	Carcinoma, bronchus	1	8*		1		10
	Carcinoma, skin		5				5
	Carcinoma, breast		6				6
	Carcinoma, tongue		4				4
	Carcinoma, adrenal cortex	2					2
	Carcinoma, gallbladder		1				1
	Carcinoma, pharynx		1				1
	Carcinoma, colon		4				4
	Carcinoma, buccal mucosa		1				1
	Carcinoma, salivary gland	1					1
	Carcinoma, ethmoid sinus		2				2
	Carcinoma, vulva		1				1
	Carcinoma, thyroid		1				1
	Carcinoma, esophagus		1				1
	Sarcoma, breast	1	1				2
	Fibrosarcoma, thigh		1	1			2
	Fibrosarcoma, mediastinum	1					1
	Fibrosarcoma, retroperitoneal	1					1
	Neurogenic sarcoma	2					2
	Osteogenic sarcoma	3	2				5
	Ewing's tumor, bone	1					1
	Giant-cell tumor, bone	2					2
	Myeloma, bone	1					1
	Synovioma, knee		1				1
	Reticuloendothelioma		1				1
	Hemangiosarcoma, pharynx		1				1
	Mixed tumor, salivary gland	1					1
	Seminoma, testis		1				1
	Malignant melanoma, skin		5			1*	6
	Neuroblastoma, mediastinum	1					1
	Neuroblastoma, adrenal	1					1
	Neuroblastoma, retroperitoneal	1					1
	Astrocytoma, brain	1					1
	Total	29	66	3	1	1	100

* One case from autopsy material.

ANIMAL TUMORS

The tumors of lower animals which were studied in standardization and control experiments during this investigation are listed in Table 3.

Group I (transplants reaching the stage of growth).—Of the 38 different animal tumors transplanted, 6 showed strong growth.

1. *Carcinoma of breast;* mouse C3H (tumor no. 1W): this tumor grew well in the anterior chamber and was used regularly in control experiments. Early outgrowth from this transplant is usually apparent at the eighth day; strong growth usually continues during the first 3 weeks. The histologic sections of the harvested tissue showed noticeable differentiation of the tumor pattern; whereas the original tumor was composed of solid sheets of cells, the tissue from the guinea pig eye showed many large glandular elements filled with clear pink-staining material.
2. *Sarcoma 37;* mouse (tumor no. 4A): this tumor grew rapidly, often filling the anterior chamber by the tenth day. The harvested tissue was much less anaplastic microscopically than the original tumor tissue.
3. *Earle fibrosarcoma;* mouse (tumor no. 6X): this tumor grows consistently in the anterior chamber. Initial outgrowth is usually evident by the tenth day. Strong growth continued during the first 3 weeks. Tissue harvested from the anterior chamber closely resembled that of the original tumor.
4. *Carcinoma of the liver;* cat (tumor no. 9W): in one transplant enlargement was evident at 34 days. Approximately one-half of the anterior chamber became filled with tumor tissue at 98 days. Tissue harvested at this time showed a cellular pattern similar to the original tumor.

TABLE 3

ANIMAL TUMORS TRANSPLANTED TO THE ANTERIOR CHAMBER OF THE EYE OF GUINEA PIGS

			SOURCE OF TISSUE			
GROUP	ANIMAL	TYPE OF TUMOR	Primary site	Lymph node metastasis	Transplant	TOTAL NO.
I	Mouse	Carcinoma, breast (C3H)			1*	1
	Mouse	Sarcoma 37			1*	1
	Mouse	Fibrosarcoma (Earle)			1*	1
	Cat	Carcinoma, liver	1			1
	Dog	Fibrosarcoma, retroperitoneal		1		1
	Cow	Carcinoma, kidney	1			1
II	Cow	Fibrosarcoma, subcutaneous	1			1
	Horse	Pheochromocytoma, adrenal	1			1
	Horse	Malignant melanoma, skin		3		3
III	Fish	Carcinoma, kidney	1			1
	Fish	Fibrosarcoma, subcutaneous	1			1
	Mouse	Carcinoma 15091A			1*	1
	Rat	Lymphosarcoma	1			1
	Rat	Granulosa-cell tumor, ovary			1†	1
	Dog	Carcinoma, breast	5			5
	Dog	Carcinoma, skin		2		2
	Dog	Carcinoma, ovary		1		1
	Dog	Osteogenic sarcoma	2			2
	Dog	Sarcoma, spleen	1			1
	Dog	Fibrosarcoma, subcutaneous	2	2		4
	Hog	Carcinoma, kidney	1			1
	Cow	Carcinoma, skin	1	1		2
	Horse	Carcinoma, thyroid	1			1
	Horse	Carcinoma, skin	1	1		2
	Horse	Fibrosarcoma, subcutaneous	1			1
		Total	22	11	5	38

* Tumor maintained by serial transplantation in the mouse.
† Tumor developed by auto-transplantation of ovary to spleen.

5. *Fibrosarcoma*, retroperitoneal; dog (tumor no. 1C): histologically, the tumor was composed of delicate bundles of fibrocytic element. The transplants in the anterior chambers of two of the eight guinea pigs filled one-third of the anterior chamber at 30 days. Tissue harvested at 118 days resembled the pattern in the original tumor histologically.
6. *Carcinoma of the kidney;* bovine (tumor no. 2T): growth became evident in the anterior chamber of one guinea pig on the 40th day; the entire chamber was filled by the 64th day. In contrast to mouse tumors which when transplanted to the guinea pig eye tended to become differentiated histologically, the tissue harvested from the anterior chamber of the guinea

pig in this case appeared less differentiated than the original tumor.

Group II (*transplants persisting in the stage of nidation*).—

1, 2, 3. *Malignant melanomas;* horse (tumors nos. 1F, 3W, 4J): three different horse melanomas on transplantation established firm nidation. Gross enlargement could not be defined. Tissue recovered after periods of 84, 32, and 22 days, respectively, showed melanoma elements resembling the original tumor.

4. *Fibrosarcoma,* subcutaneous; cow (tumor no. 3K): tissue harvested from one transplant after 13 days showed persisting tumor elements histologically.

5. *Pheochromocytoma of adrenal;* horse (tumor no. 3Z): tissue harvested from one transplant after 88 days showed well preserved tumor elements.

Group III (*transplants undergoing early regression*).—Of the 38 animal tumors transplanted, 27 showed regression during the stage suspension. These tumors are listed in Table 3.

DISCUSSION

Since only 2 per cent of the human malignant tumors showed definite growth when transplanted to the guinea pig eye, and since growth in each instance was preceded by a long latent period, it is evident that the present procedure cannot be applied as a diagnostic measure to differentiate benign from malignant tumors. It is apparent that most human neoplasms do not possess sufficient growth momentum to overcome the barrier of species difference involved in heterologous transplantation to the guinea pig. The transplantation experiments with tumors of lower animals showed that, whereas the mouse tumors investigated grow readily in the guinea pig eye, comparable tumors from animals of higher species grow less often when transplanted to the guinea pig; this indicates the importance of the degree of species difference in transplantability of heterologous tumors. In order that the proposed technic may be used in the diagnosis of human tumors, modifications must be devised to reduce the handicap of the species barrier.

SUMMARY

It has been proposed by previous investigators that heterologous transplantation of human tumors to the guinea pig eye be used as the basis for differentiating benign from malignant growths. This procedure is based upon the premise that a significant number of human malignant tumors have sufficient growth momentum to overcome the barrier of species difference involved in heterologous transplantation. The present study is an evaluation of the clinical applicability of the proposed technic as a diagnostic measure. In the preliminary phase of the investigation, principles of procedure and criteria for evaluation were outlined.

Studies have indicated that the establishment of a transplant in the anterior chamber evolves through three well defined phases: first, the suspension stage, during which the transplant exists in a "tissue culture" state; second, the nidation stage, during which the transplant becomes attached and vascularized; and third, the growth stage, during which active proliferation of the tumor elements is manifested. In evaluating results tumors were classified in groups according to the maximal stage of development attained by transplants.

One hundred different human malignant tumors were studied. Two tumors, comprising Group I, showed active growth after long latent periods in the anterior chamber; one was a fibrosarcoma, the second was a carcinoma of the maxillary sinus. Transplants of nine tumors, comprising Group II, persisted for periods ranging from 10 to 114 days in the stage of nidation. Eighty-nine of the tumors, comprising Group III, showed early regression of transplanted tissue.

Of the 38 animal tumors studied, 6 showed growth, 5 showed persistence from 13 to 88 days of the transplants in the stage of nidation, and 27 underwent early regression. These results support the premise that heterologous transplantation is more easily accomplished when the degree of species difference is decreased.

The results of this investigation indicate that relatively few human tumors, though clinically malignant, can be transplanted successfully to the anterior chamber of the guinea pig eye; this factor precludes the use of this technic as a practical measure for differentiating benign from malignant tumors.

ACKNOWLEDGMENTS

The illustrations were prepared by Joe D. Humphrey, Department of Pathology, Ohio State University, College of Medicine.

REFERENCES

1. GREENE, H. S. N. Identification of Malignant Tissues. J.A.M.A., **137**:1364–66, 1948.
2. GREENE, H. S. N., and LUND, P. K. The Heterologous Transplantation of Human Cancers. Cancer Research, **4**:352–63, 1944.
3. MORRIS, D. S.; MCDONALD, J. R.; and MANN, F. C. Intraocular Transplantation of Heterologous Tissues. Cancer Research, **10**:36–48, 1950.
4. NEWTON, B. L. Differentiation of Benign and Malignant Tumors by Heterotransplantation. Texas State J. Med., **46**:252–53, 1950.
5. SCHILLING, J. A.; SNELL, A. C., JR.; and FAVATA, B. V. Heterologous Ocular Transplantation as a Practical Test for Cancer. Cancer, **2**:480–89, 1949.
6. TOWBIN, A. A Study of Tumor Tissue after Transplantation. Arch. Path. (in press).

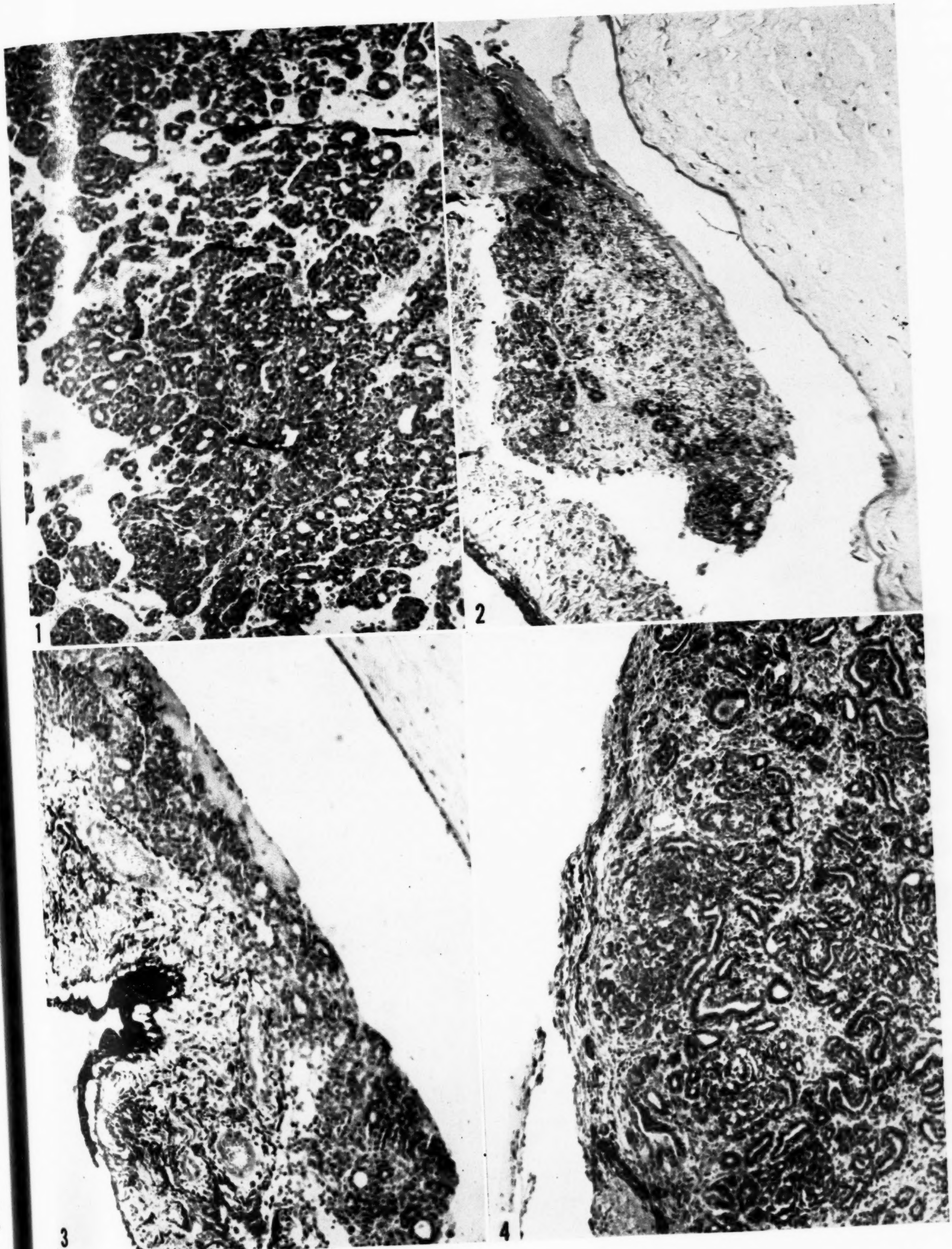

1
2
3
4

FIG. 1.—Mammary carcinoma from the C3H mouse. ×70.

FIG. 2.—Stage of suspension. Mammary carcinoma (C3H mouse) 2 days after transplantation to the anterior chamber of the eye of the guinea pig. The transplant lies loosely between the iris and the cornea. ×70.

FIG. 3.—Stage of nidation. Mammary carcinoma (C3H mouse) 4 days after transplantation to the anterior chamber of the eye of the guinea pig. The transplant is firmly attached to the pigmented iris and is being vascularized. ×70.

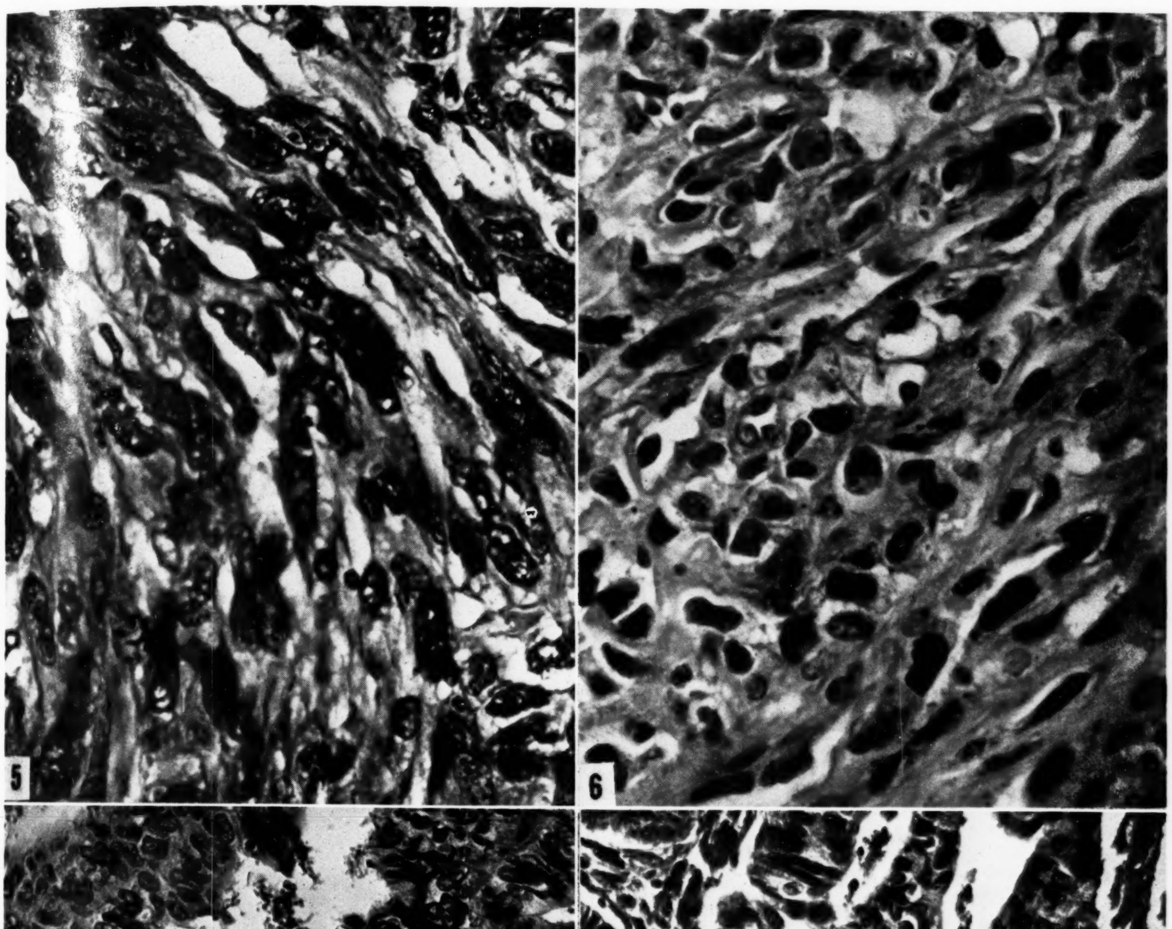
FIG. 4.—Stage of growth. Mammary carcinoma (C3H mouse) 10 days after transplantation to the anterior chamber of the eye of the guinea pig. The transplanted tissue, growing outward from the iris, shows large, well differentiated glandular elements. ×70.

FIG. 5.—Fibrosarcoma; metastasis to the lung from the primary site in the arm; human (tumor no. 6Y). ×650.

FIG. 6.—Transplant of fibrosarcoma (tumor no. 6Y). Growth stage in the guinea pig eye. ×650.

FIG. 7.—Carcinoma of maxillary sinus; human (tumor no. 9T). ×650.

FIG. 8.—Transplant of carcinoma of maxillary sinus (tumor no. 9T). Growth stage in the guinea pig eye. ×650.


5
6
7
8

The Effect of Mammary Tumors on the Glucuronidase and Esterase Activities in a Number of Mouse Strains*

SAUL L. COHEN AND JOHN J. BITTNER

(Department of Physiological Chemistry, the Max H. Hoffman Endocrinological Research Laboratory, and the Division of Cancer Biology, Department of Physiology, University of Minnesota Medical School, Minneapolis, Minn.)

The reports of Fishman and Anlyan (4) and of Odell and Burt (12) indicating a high β-glucuronidase activity in a number of human cancerous tissues have elicited considerable interest in the possibility of a relationship between this enzyme and neoplasms in general. The lack of species specificity for such a relationship is indicated in the observation by Kerr *et al.* (9) of a higher glucuronidase concentration in mammary tumors than in the normal tissue of mice (of undesignated strains).

Harris and Cohen (8) observed an inverse relationship between changes in β-glucuronidase and esterase activity levels for a number of tissues of mice subjected to variations in the amount of circulating sex hormones. Similar inverse changes between these enzymes have also been reported (2) as occurring in the sera of patients with breast cancer on estrogen therapy. The investigations reported in this paper were undertaken in part to determine whether a similar inverse relationship might occur in the mammary tumors of inbred mice. Such a possibility seemed supported by the report of Greenstein in 1944 that tumors of the liver, lymph node, and intestine in a number of strains of mice possessed a considerably lower esterase concentration than the corresponding normal tissue.

A second aspect of the studies reported herein was to determine whether the glucuronidase and esterase activities in the mammary tissues varied for different strains of mice. A number of reports have appeared within the last decade on variations in enzyme patterns in a variety of inbred mouse strains. In 1942 Khanolkar and Chitre reported that the serum esterase activity of C57 mice was lower than for the C3H and A strains. Subsequently, Shimkin, Greenstein, and Andervont (13) noted a lack of correlation between serum esterase and susceptibility to mammary tumors. Thus, they observed that the C and I strains showed serum esterase activities which were, respectively, the same as and greater than those found in the mice of the C3H strain. Many studies have also been carried out on the livers of inbred mice with tumors, and certain strain variations have been reported for the enzymes xanthine dehydrogenase (3, 6), liver catalase (5), and glucuronidase (11). No strain differences have been observed for a large number of other hepatic enzymes studied (for references see 11).

These two problems are answered, at least in part, by the data reported in this paper, which is based on studies on the glucuronidase and esterase activities of the tumorous and nontumorous mammary tissue of a number of strains of mice.

METHODS

Mouse strains.—A total of 69 mice was used in these experiments. Animals with mammary cancer were from the following stocks or F_1 generations: the Andervont subline of the C3H stock; the author's (J. J. B.) line of the C3H stock referred to as the Z strain; D_8 subline of the D or dilute brown stock; ZD_8F_1 hybrids (Z ♀ $\times$ D_8 ♂); A; $AJKF_1$ (A ♀ $\times$ JK ♂); and C stock. Animals of five strains, Ax, Zb, JK, C57 black (sublines 1 and 4), and C, were without the milk agent and therefore without mammary tumors (1). Of these latter groups, the Ax, Zb, and C mice were susceptible to the development of spontaneous mammary cancer. All the females employed in these studies were nonpregnant and averaged 13 (8–17) months of age. For each strain three to twelve mice were used.

* This work was aided by grants from the American Cancer Society through an institutional grant made to the University of Minnesota, and upon recommendation of the Committee on Growth of the National Research Council; a grant from the National Cancer Institute, Public Health Service; and grants from the University of Minnesota Graduate School.

Received for publication May 16, 1951.

Preparation of tissues.—Most of the animals were killed with ether; those animals on which blood enzyme assays were also carried out were killed by cutting the jugular veins. The mammary tissue was immediately removed, the tumorous and nontumorous tissue separately pooled and chilled. The tissues were weighed and prepared for assay by procedures already described (8).

Enzyme assays.—The glucuronidase and esterase (butyrate) activities of the tissues were determined by the methods outlined by Harris and Cohen (8). The serum enzymes were determined by procedures slightly modified from those of Cohen and Huseby (2). A unit of enzyme is defined as the amount of enzyme which liberates 1 μg. per hour of phenolphthalein and butyric acid from their corresponding substrates.

To obviate the diluting effects of water and of lipids, nitrogen determinations were carried out on aliquots of each homogenate, and the tissue enzyme activities reported in this paper are expressed in terms of the nitrogen content of the tissue, rather than on a basis of tissue wet weight.

RESULTS AND DISCUSSION

The results of the enzyme determinations for mouse mammary tissues are summarized in Table 1. The following observations may be made:

A. β-Glucuronidase activities

1. Marked variations occur in the glucuronidase activity of the mammary tissue of different strains of mice. The lowest nontumorous value for the mice with the milk agent was found for the Andervont C3H mice (4.0 ± 0.47), while the highest values were observed for the $AJKF_1$ (112 ± 13.1) and for the C (119 ± 7.9) strains. The Z mice (Bittner C3H line) were also found to possess a low mammary glucuronidase level (25 ± 3.4). A significant difference for the two sublines of the C3H stock (Andervont and Bittner sublines) is, however, to be noted. No significant differences were observed for ZD_8F_1, D_8, and A mice (average glucuronidase activity about 88 units).

2. Similar strain differences were also observed for the glucuronidase activities of mice without the milk agent and without spontaneous mammary tumors. Of the five such strains examined, an increasing order of enzyme activity was found for the Zb (43 ± 4.5), JK, C, and Ax (125 ± 7.8) mice. It is also to be noted that for strains A and Z the presence of the milk agent results in a considerable decrease in the glucuronidase activity of the nontumorous mammary tissue, while for the C strain the mammary tissue glucuronidase level was higher than that in animals possessing the milk agent. It is possible that this difference is due to the fact that the C mice received the milk agent relatively late in life.

3. There are indications that the glucuronidase activity levels for the different strains of animals are genetically determined. This is particularly indicated in the mammary tissue values obtained for the ZD_8F_1 strain of mice in which the glucuronidase levels are about the same as for the D_8 mice but about 3–4 times as great as for the Z mice. Further support for the hypothesis of genetic control of glucuronidase levels is seen in the data (summarized in Table 2) obtained for enzyme assays on blood sera. It is seen that the serum glucuronidase of the Z mice is only about 20 per cent as high as that of the A strain, while the glucuroni-

TABLE 1

GLUCURONIDASE AND ESTERASE ACTIVITY LEVELS IN MOUSE MAMMARY TISSUE

		Glucuronidase units/mg tissue N			Esterase units/mg tissue N		
		(a)	(b)	(c)	(d)	(e)	(f)
Strain	No. of mice	Normal	Cancerous	Ratio $\frac{b}{a}$	Normal	Cancerous	Ratio $\frac{d}{e}$
C3H	3	4± 0.47	51±12.6	13	11,500±2,300	4,600±1,000	2.5
Z	11	25± 3.4	70±21.6	3	16,100±3,900	4,400±1,000	4
ZD_8F_1	7	82±11.8	170±24.5	2	21,000±3,300	1,800± 200	12
D_8	4	92± 9.3	174±21.5	1.9	15,000±3,700	1,700± 580	8
A	8	89± 9.6	156±12.2	1.8	13,000±1,900	5,000± 580	2.5
$AJKF_1$	3	112±13.1	191±23.1	1.7	10,500±1,700	1,170± 350	9
C	3	119± 7.9	194±74.2	1.6	8,850±1,400	1,150± 280	7.7
Zb	5	43± 4.5			15,000±1,600		
JK	5	60±15			24,200±3,800		
C57 black, line 1	6	72±12			14,800±2,270		
C57 black, line 4	4	78±10.6			13,900±1,730		
C	4	76±13.7			17,000±1,600		
Ax	4	125± 7.8			11,300± 770		

All values are expressed as averages ± the standard error.

dase activity of the nontumorous mammary tissue for the Z strain was about 28 per cent of that of the A strain. In this connection it might also be pointed out that Morrow *et al.* (11) reported the liver glucuronidase activity of Andervont C3H mice to be only about 10 per cent of that of A mice, which is comparable to our findings of about 5 per cent for nontumorous mammary tissue. The final proof of a possible genetic determination of the glucuronidase activity in mice must await assays on a large number of mice in experiments specifically designed for this purpose.

4. An increased β-glucuronidase activity of the malignant mammary tissue, as compared to that of the nonmalignant mammary tissue, was observed for all strains of mice examined. Indeed, no exception to this relationship has been found in any of the 42 mice with mammary tumors thus examined in our laboratory. It can also be seen that a similar species difference is demonstrated for the cancerous mammary tissue, as for the corresponding noncancerous tissues. The percentage increase in glucuronidase activity, however (see Table 1, column c), varies inversely with the relative levels in the noncarcinogenic tissue. Thus, in mice of three strains, the cancer tissue in the C3H strain showed 1300 per cent as much glucuronidase as the noncancer tissue, while corresponding values of nearly 300 per cent for the Z stock and of only 160 per cent for the C mice were shown.

B. Esterase (butyrate) activities

1. A much smaller percentage variation in the esterase content of nontumorous tissues was noted among different mouse strains than was found for the β-glucuronidase activities. A maximum spread of about $2\frac{1}{2}$-fold was found for the strains examined (the C animals with the milk agent showed the lowest values, while the ZD_8F_1 mice with the agent and JK group without the agent had the highest esterase activities). There seems to be no uniform relationship between the relative glucuronidase and esterase activity levels in the nontumorous mammary tissue of different strains of mice.

A lack of relationship between the relative glucuronidase and esterase levels of different mouse strains is also to be seen in the serum assays (Table 2). Thus, while the Z and C57 mice showed approximately the same serum esterase levels, the serum glucuronidase of the former strain was about $\frac{1}{3}$ that of the latter group of mice. These data also confirm the previous reports of others (10, 13) that the C57 mice have a lower serum esterase activity than that of the A strain.

2. The presence of the milk agent had no significant effect on the esterase content of nontumorous mammary tissue of the A or Z strains but was associated with a marked decrease (about 50 per cent) for the C strain.

3. In all the mice examined, the neoplastic mammary tissue had a markedly lower esterase activity than did the noninvolved mammary tissue. It is also of interest that, with the exception of the A mice, there seems to be an inverse relationship between the esterase level of the mammary cancer tissue and an approximately direct one for the ratio of noncancer tissue to cancer tissue esterase activities (see Table 1, column f), and the relative glucuronidase levels for the different strains of mice.

TABLE 2

SERUM ESTERASE AND GLUCURONIDASE LEVELS IN MICE

STRAIN	NO. OF ASSAYS	GLUCURONIDASE Units/100 cc serum	ESTERASE Units/cc serum
Z	4	363±111	26,700±5,900
C3H	1	718	32,100
C57, line 1	3	1,120± 88.5	25,600±2,630
C57, line 4	3	1,180± 44.2	27,100±1,490
A	8	1,950±193	37,700±2,520
C	1		55,200

All values are expressed as averages ± the standard error.

4. While no proof for the discrepancy shown by the A strain in the above relationships is at present available, it is possible that this may be related to the lipid content of the mammary tissue. Thus, in a preliminary experiment the lipid (the petroleum ether–soluble fraction) content of the mammary tissue has been observed to be about $\frac{1}{5}$ as great for cancerous as for noninvolved tissue for mice of both the A and C3H strains. For both the involved and noninvolved mammary tissues, however, the lipid content was about 3 times as high for the A strain (0.67 ± 0.36 per cent and 3.1 ± 1.3 per cent, respectively) than for the C3H strain (0.27 ± 0.06 per cent and 1.3 per cent, respectively). It is entirely possible that some direct relationship exists between the lipid content and esterase activity of a tissue. Thus, a relatively low esterase level effected by one set of circumstances might be masked by a high esterase activity related to another set of circumstances. Such an explanation might also explain the apparent lack of consistency for the esterase levels referred to in sections *B*, 1 and 2, above.

5. An increased glucuronidase and decreased esterase have been observed for all neoplastic mammary tissues, as compared to the corresponding uninvolved tissues examined. This inverse relationship between glucuronidase and esterase changes is similar to that previously reported for other experiments (2, 8).

There appears to be no relationship between mammary glucuronidase and esterase activities and tumor incidence in the mice studied. Thus, for example, both the glucuronidase and esterase activities of the C3H strain (possessing high tumor incidence) are lower than those of the C and JK strains, but the C mice are also a strain of high tumor incidence when they possess the milk agent, while the JK mice are a strain showing a low tumor incidence under similar circumstances.

SUMMARY

1. The results of β-glucuronidase and esterase determinations for the cancerous and uninvolved mammary tissue of 39 mice of 7 different strains with the milk agent and of 30 mice of 5 strains without the agent are reported.

2. Considerable variation in the β-glucuronidase levels was found for the various strains of mice both with and without the milk agent. Of the animals possessing tumors, the C3H strain of mice showed only 3.3 per cent as much glucuronidase activity as did the C and $AJKF_1$ strains, with the Z, ZD_8F_1, D_8, and A strains possessing values between these extremes.

3. Evidence of a genetic influence on the glucuronidase activity levels of different mouse strains is presented and discussed.

4. Variations in the esterase levels of the cancerous mammary tissues were found in most instances to vary inversely with the relative mammary glucuronidase activities of the various strains of mice examined. A possible explanation for the one exception (A mice) in this group, as well as the apparent small and unrelated variations in the esterase levels of the uninvolved mammary tissues, is discussed.

5. In all the animals examined, tumors of the mammary glands were associated with increased β-glucuronidase and decreased esterase activities, as compared to those of uninvolved mammary tissue. In most cases the per cent difference between the involved and uninvolved tissue varied inversely for the glucuronidase and directly for the esterase activities with the relative glucuronidase levels of the different strains of mice.

6. The variations in β-glucuronidase and esterase activity levels are apparently not related to the tumor incidence in the various strains of mice.

ACKNOWLEDGMENTS

We wish to make grateful acknowledgment to Ruth S. Harris, Shirley C. Tennyson, and Florence I. Bowers for carrying out the assays involved in these studies.

REFERENCES

1. Bittner, J. J. Some Enigmas Associated with the Genesis of Mammary Cancer in Mice. Cancer Research, **8**:625–39, 1948.
2. Cohen, S. L., and Huseby, R. Inverse Changes in Serum Glucuronidase and Esterase of Breast Cancer Patients on Estrogen Therapy. Proc. Soc. Exper. Biol. & Med., **76**: 304–7, 1951.
3. Figge, F. H. J., and Strong, L. C. Xanthine Oxidase (Dehydrogenase) Activity in Livers of Mice of Cancer-susceptible and Cancer-resistant Strains. Cancer Research, **1**:779–84, 1941.
4. Fishman, W. H., and Anlyan, A. J. β-Glucuronidase Activity in Human Tissues. Some Correlations with Processes of Malignant Growth and with the Physiology of Reproduction. Cancer Research, **7**:808–17, 1947.
5. Greenstein, J. P. The Liver Catalase Activity of Tumor-bearing Mice and the Effect of Spontaneous Regression and of Removal of Certain Tumors. J. Nat. Cancer Inst., **2**:345–55, 1942.
6. ———. Range in Activity of Several Enzymes in Normal and Neoplastic Tissues of Mice. *Ibid.*, **4**:275–81, 1943.
7. ———. Esterase (Butyric Esterase) Activity of Normal and Neoplastic Tissues of the Mouse. *Ibid.*, **5**:31–34, 1944.
8. Harris, R. S., and Cohen, S. L. The Influence of Ovarian Hormones on the Enzymic Activities of Tissues. Endocrinology, **48**:264–72, 1951.
9. Kerr, L. M. H.; Campbell, J. G.; and Levvy, G. A. Further Observations on Changes in β-Glucuronidase Activity in the Mouse. Biochem. J., **46**:278–84, 1950.
10. Khanolkar, V. R., and Chitre, R. G. Studies in Esterase (Butyric) Activity. I. Esterase Content of Serum of Mice from Certain Cancer-Resistant and Cancer-Susceptible Strains. Cancer Research, **2**:567–70, 1942.
11. Morrow, A. G.; Greenspan, A. M.; and Carroll, D. M. Liver-Glucuronidase Activity of Inbred Mouse Strains. J. Nat. Cancer Inst., **10**:657–61, 1949.
12. Odell, L. D., and Burt, J. C. Beta-Glucuronidase Activity in Human Female Genital Cancer. Cancer Research, **9**:362–65, 1949.
13. Shimkin, M. B.; Greenstein, J. P.; and Andervont, H. B. Esterase Activity of Blood Serum of Four Strains of Mice. J. Nat. Cancer Inst., **5**:29–30, 1944.

The Effect of Low Temperatures on Serial Transplantability of Mouse Sarcoma 37*

LAWRENCE B. WALSH, DONALD GREIFF, AND H. T. BLUMENTHAL

(Departments of Biology and Pathology, St. Louis University School of Medicine, and the Department of Pathology of The Jewish Hospital, St. Louis, Mo.)

The recent investigations of Gye and associates (5–7, 11, 12), dealing with the successful transfer of frozen and frozen-dehydrated tumor tissue, have led to a re-evaluation of this technic as a means of demonstrating a virus or virus-like factor in the transmission of neoplasms. Certain aspects of this subject have been dealt with by us in previous reports (3, 4, 13), and several other investigators (1, 8) have also recently considered various other aspects of this problem.

Two observations in our previous experiments (13) appear to be particularly inconsistent with the behavior of a virus: (*a*) the loss of the growth potential of the tumor following repeated freezing and thawing and (*b*) the sudden inability of frozen tumor to grow after several serial transplantations without further exposure to low temperature. Additional observations concerning this latter phenomenon are reported here, utilizing the PR8 strain of Influenza A virus for the purpose of comparing the response of a known virus with tumor tissues.

MATERIALS AND METHODS

Sarcoma 37 was used, as in previous investigations (13); it has been shown that this tumor has a low strain specificity and has now been carried in an unfrozen state through many transplant generations in four different strains used in this laboratory, as well as between these various strains; the latter are St. L. U., Rockland, CAF-1, and CFW. Exposures to various low temperatures were carried out in a manner described previously, but storage was for only 24 hours, except in the case of Influenza A virus.

Chorioallantoic fluid infected with Influenza A virus was stored at −30° C. several times during the course of serial transfer, as shown in Table 2. Infectivity titers were determined according to a method previously described (2), in order to note any change in potency of the virus resulting from storage at low temperatures.

RESULTS

The data in Table 1 show a striking cessation of tumor growth following the fourth serial transplantation of this tumor after a single freezing and thawing. The variations in latent period are not significant, since a similar variability has been demonstrated for unfrozen tumor in our previous report. Tiny nodules could be palpated at the site of transplantation for several days after the fourth serial transplantation had been accomplished, but these gradually disappeared completely during a subsequent period of approximately 1 week.

TABLE 1

SARCOMA 37 STORED AT −70° C. FOR 24 HOURS

Transplant generation	No. of mice	Strain	Results: +	Results: −	Per cent "takes"	Latent period (days)
1	19	Rockland	18	1	95	10
2	10	CAF-1	10	0	100	8
3	10	"	8	2	80	15
4	10	Rockland	0	10	0	

The experiment shown in Table 1 represents the sixth time we were able to demonstrate this phenomena; it is characteristic of the end result in every instance in which serial transplantation was attempted. This includes three experiments in which serial transfer was limited to a single strain, but in each instance a different strain was used; it also includes three experiments in which combinations of strains were utilized. In addition, two other investigators utilized our frozen tumor material for unrelated investigations and observed the same phenomenon.[1,2] Of these eight observations, five were with tumor stored at −30° C., and

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service.

Received for publication May 18, 1951.

[1] D. Harris, personal communication.

[2] L. P. Laskowski, personal communication.

three with tumor stored at $-70°$ C. Storage periods have varied from 24 hours to approximately 3 months. The longest period of survival was through seven transplant generations; in the latter experiment CAF-1 mice received tumor which had been stored for 7 days at $-30°$ C.

In contrast to the results obtained with tumor tissue, Influenza A virus frozen at $-30°$ C. can apparently be transferred through many generations without significant loss of potency. Nor does the duration of storage or repeated freezing and thawing appear to have any appreciable effect on the infectivity titer. Data illustrating these points are shown in Table 2.

TABLE 2

INFLUENZA A VIRUS STORED AT $-30°$ C. FOR VARYING PERIODS

Transplant generation	Treatment	Infectivity titer*
1	Unfrozen	−8.0
2	"	−7.2
3	"	−8.4
4	"	−8.4
5	"	−7.8
6	−30° C., 7 days	−8.0
7	" 5 weeks	−7.2
8	" 12 "	−7.0
9	" 18 "	−8.8
10	" 6 months	ND†
11	Unfrozen	ND
12	"	ND
13	"	ND
14	"	ND
15	"	−7.8

* Figures in this column represent only exponents. For example, complete figure would be $10^{-8.0}$, $10^{-7.2}$, etc.

† ND represents no determination.

In contrast to the results obtained with Sarcoma 37 frozen at $-30°$ C. or $-70°$ C., tumor placed in liquid nitrogen for 15 minutes before storage at $-70°$ C. can be successfully carried through many transplant generations. Data substantiating this conclusion are shown in Table 3. The variation in time between implantation and first appearance of the tumor (latent period) is again within the limits seen following transplantation of unfrozen tumor mince, as are also the variations in percentage "takes." The low percentage of takes in the first transplant generation is probably caused by technical error.

DISCUSSION

These observations, then, confirm and extend those noted previously (13). Sarcoma 37 frozen at $-30°$ C. or $-70°$ C., and subsequently serially transplanted, fails to proliferate after three to seven transplant generations. Likewise, as reported previously, multiple freezing and thawing also cause a cessation of tumor growth. In contrast, Influenza A virus suspended in chorioallantoic fluid can be transferred serially for an indefinite period without apparent loss of infectivity, despite the interjection of numerous exposures to freezing at $-30°$ C. and subsequent thawing. The duration of storage at low temperature appears to be without influence on these phenomena. On the other hand, tumor mince frozen relatively rapidly at $-190°$ C. before storage at $-70°$ C. behaves in a manner similar to virus, in that such neoplastic tissue can also be serially transferred indefinitely; multiple exposures of this type have not been tested to date.

Previously, we had pointed out the importance of the rate of freezing in protecting normal and neoplastic cells from the injurious effects of ice crystal formation; reported differences in experiments with tumors on the effects of rapid and slow freezing represented only different rates of slow freezing. In none of the latter investigations did the rate of freezing approach several hundred degrees per second, which Luyet and Gehenio (10) have shown to be necessary to obtain an intracellular vitreous state; the experiment shown in Table 3 in which tumor was first placed in liquid nitrogen probably represents the nearest approach to such a rate of freezing.

TABLE 3

SARCOMA 37 PLACED AT $-190°$ C. FOR 15 MINUTES AND THEN AT $-70°$ C. FOR 24 HOURS

			Results			
Transplant generation	No. of mice	Strain	+	−	Per cent "takes"	Latent period (days)
1	19	Rockland	7	12	37	10
2	10	CAF-1	10	0	100	8
3	10	"	10	0	100	8
4	11	"	11	0	100	9
5	5	Rockland	5	0	100	10
6	5	CAF-1	5	0	100	11
7	5	"	5	0	100	7
8	5	"	4	1	80	7
9	5	"	4	1	80	7
10	5	"	5	0	100	10
11	5	"	5	0	100	7
12	10	"	10	0	100	14

The behavior of tumor tissue after freezing-thawing is therefore different from the behavior of a typical infectious virus. It resembles virus in behavior only when conditions of freezing are such as to minimize injury to tumor cells. These experiments further indicate that the injurious effects may not appear immediately, but may become manifest only after several transplant generations. In this respect, the injurious effects seem to be somewhat different from those more immediately produced by various chemical and biological

agents. However, we wish to point out again the desirability of carrying tumor, apparently unsuccessfully treated with such agents, through several transplant generations before reaching a conclusion as to an injurious or destructive effect.

The mechanism of this delayed manifestation of cell damage is not apparent from these observations, although several possibilities deserve consideration.

1. Relatively slow freezing may produce a somatic mutation incompatible with continued life of the cell. As far as we have been able to determine, such an alteration has not been demonstrated, nor, in the event of development of a lethal factor, is it likely to require a number of transplant generations before becoming manifest. On the contrary, we have observed cells of frozen-thawed thyroid autotransplants in the guinea pig which respond to thyrotropin stimulation with apparently normal mitotic processes (3). The mechanism of injury of cells in freezing-thawing experiments appears to be related to the formation of ice crystals; chromosomal abnormalities have not been reported.

2. The frozen-thawed cell may develop an antigenicity not resident in the unfrozen tumor cell, to which host animals can develop antibodies in a relatively short time. Such antibodies could conceivably enter tumor cells and build up a sufficient concentration to prove lethal only after several transplant generations.

3. There is also the possibility that freezing-thawing causes destruction of certain critical enzyme systems which may be necessary for the removal of injurious metabolites. A sufficient accumulation of such metabolites to prove lethal to tumor cells may necessitate a period longer than that necessary for tumor to kill several of its hosts and therefore would become manifest only after several serial transfers.

4. On the other hand, these observations may also be interpreted as resulting from the destruction of a stimulating or virus-like agent, following which the tumor is able to maintain an autonomous growth for only several subsequent transplant generations. In support of such a thesis are the observations of Jensen (9) on the growth of tumors in beets and mangels induced by *Phytomonas tumefaciens*. In these plants the tumor persisted and could be successfully transplanted even after the bacteria had apparently disappeared. While such tumor transplants grew effectively for three or four generations, the abnormal proliferative power eventually disappeared. The validity of such an interpretation would rest upon the demonstration of a continuing stimulating agent in tumor cells, and would therefore tend to disprove the concept of tumor as an autonomous growth.

The present experiments are therefore pertinent to a critical evaluation of the concept of tumor as an autonomous growth. In the case of some chemically and hormonally induced tumors it is known that, even when the growth stimulus is removed before the tumor develops, neoplasms subsequently occur, and such data tend to support the autonomy hypothesis. On the other hand, in the case of mammary tumors in mice and possibly in some other tumors, the "milk factor" may constitute a continuing stimulus. In the case of the Rous sarcoma of the chicken and other virus tumors, the virus may represent both the initiating and continuing stimuli. However, a continuing factor has not been demonstrated in either human cancer or in the vast majority of animal tumors.

From our observations, it does not appear likely that a viral agent in the same sense as that used to define a sub-microscopic infectious agent can be implicated as an initiating agent to the development of mouse Sarcoma 37, but some stimulating agent may be required for the apparent autonomous growth of this tumor.

The concept of initiating and continuing factors in neoplastic growth is, of course, not a new one. If the present observations are to be cited in support of such a concept, then it must also be concluded that, unlike the effect on a virus, freezing-thawing destroys rather than preserves such an agent, except probably under conditions which also preserve the integrity of the cell.

SUMMARY

The present experiments confirm and extend those reported previously. Mouse Sarcoma 37, frozen at $-30°$ or $-70°$ C. and subsequently serially transplanted, fails to grow after three to seven transplant generations. This phenomenon occurs both when transplantations are carried out in a single strain or between various strains. On the other hand, tumor mince frozen rapidly at $-190°$ C. before storage at $-70°$ C. can be serially transferred through numerous transplant generations without apparent loss of growth potential.

Influenza A virus suspended in chorioallantoic fluid can be transferred serially in the chorioallantoic cavity of the chick embryo for an indefinite period without apparent loss of infectivity, despite the interjection of numerous exposures to freezing at $-30°$ C. and subsequent thawing. This is in contrast to the effect on mouse Sarcoma 37, noted above, as well as to the injurious effect of repeated freezing and thawing on the growth of this tumor.

Possible mechanisms to explain the effect on serial transplantability are discussed, as is also the relationship of this finding to the demonstration of initiating and continuing factors in neoplastic growth.

REFERENCES

1. Bittner, J. J., and Imagawa, D. T. Assay of Frozen Mouse Mammary Carcinoma for the Mammary Tumor Milk Agent. Cancer Research, **10**:739–50, 1950.
2. Blumenthal, H. T.; Greiff, D.; Pinkerton, H.; and DeWitt, R. Influenza: I. The Hemagglutination and Infectivity Titre Curves of PR8 Influenza Virus Cultivated in Embryonated Eggs at Different Temperatures. J. Exper. Med., **91**:321–29, 1950.
3. Blumenthal, H. T., and Walsh, L. B. Survival of Guinea Pig Thyroid and Parathyroid Autotransplants Previously Subjected to Extremely Low Temperatures. Proc. Soc. Exper. Biol. & Med., **73**:62–67, 1950.
4. Blumenthal, H. T.; Walsh, L. B.; and Greiff, D. Studies on the Effect of Low Temperature on the Transmission of Normal and Neoplastic Tissue. Cancer Research, **10**:205, 1950.
5. Craigie, J. Director's Report. 47th Annual Report, Imperial Cancer Research Fund, pp. 5–18, 1949–50.
6. Gye, W. R. The Propagation of Mouse Tumors by Means of Dried Tissue. Brit. M. J., **1**:511–15, 1949.
7. Gye, W. R.; Begg, A. M.; Mann, I.; and Craigie, J. The Survival Activity of Mouse Sarcoma after Freezing and Drying. Brit. J. Cancer, **3**:259–67, 1949.
8. Hirschberg, E., and Rusch, H. P. Comments on Recent Experiments with Frozen and Dried Tissue as Evidence for the Virus Etiology of Tumors. Cancer Research, **10**:335–38, 1950.
9. Jensen, C. O. Investigations upon Tumor-like Formations in Plants. English Summary. Aarsskrift, pp. 91–143, 1918.
10. Luyet, B. J., and Gehenio, P. M. The Mechanism of Injury and Death at Low Temperature. Biodynamica, **3**:33–99, 1940.
11. Mann, K. Effect of Repeated Freezing and Thawing on Mouse Carcinoma Tissue. Brit. M. J., **2**:253–55, 1949.
12. Mann, I., and Dunn, W. Propagation of Mouse Carcinoma Tissue by Dried Tumor Tissue. Brit. M. J., **2**:255–57, 1949.
13. Walsh, L. B.; Greiff, D.; and Blumenthal, H. T. The Effect of Low Temperature on the Morphology and Transplantability of Sarcoma 37. Cancer Research, **10**:726–36, 1950.

The Energy Expenditure of Rats Bearing Walker Carcinoma 256*

G. B. Mider, L. D. Fenninger, F. L. Haven, and J. J. Morton

(*Division of Cancer Research, Departments of Surgery, Medicine, and Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.*)

Progressive growth of the Walker carcinoma 256 produces a caloric deficit in young adult, male, albino rats. The excessive expenditure of calories is not due solely to decreased food intake, for tumor-bearing rats lose more calories than do pair-fed noncancerous rats of the same age, sex, and initial weight. This result was anticipated by a previous report that rats in which the Walker tumor grew progressively lost more total lipid than did pair-fed control rats of the same age, weight, and sex (3). It seemed advisable, however, to determine the over-all caloric deficit acquired during the growth of this neoplasm.

MATERIALS AND METHODS

The experiments reported herein are based on two facts established by Rubner: (*a*) the law of conservation of energy holds true for the animal body (6) and (*b*) heat production by fat and carbohydrate is the same whether burned within or outside of the animal body, although the physiological heat value for protein is lower than the combustion value, because tissues burn protein incompletely (5). Thus, a caloric balance may be calculated if one determines the total calories in the animal body at the beginning and end of the period of observation and the caloric values of the ingesta and excreta during the same period.

The original experiment was conducted on seven groups of three young male rats (Carworth) of the same age and weight, housed in individually suspended, basket-type metabolism cages. They were fed a semisynthetic diet adequate for growth, pregnancy, and lactation. It had the following composition by weight:

Casein, vitamin free	25
Sucrose	63
Crisco	8
Hubbell's inorganic salts	4
	100

The following mixture of vitamins was added to each 100 gm. of diet:

Thiamin hydrochloride	0.001
Pyridoxine hydrochloride	0.001
Riboflavin	0.002
Inositol	0.005
Niacin	0.010
Calcium pantothenate	0.010
Choline chloride	0.100

One of the rats in each group, called the experimental animal, was allowed to eat the diet freely. Each of the two other rats in the group was fed the amount of the diet consumed by the experimental rat during the preceding period. Water was available at all times.

The rats were observed for a minimum period of 2 weeks to insure that they grew at the same rate while eating the same amount of food. Then a fragment of Walker carcinoma 256 was introduced aseptically by trocar into the subcutaneous tissues of the inguinal region of the experimental animal. One of the pair-fed normal control rats was killed on the day that a palpable mass appeared at the site of tumor implantation in the experimental animal of the same group. The cancerous rat was allowed to die or was decapitated when the tumor had attained a suitable size. The remaining noncancerous pair-fed rat was killed at the appropriate interval to insure identical food intake.

Urine and feces were collected separately thrice weekly. The samples obtained in 1 week from each rat were pooled for analysis. An aliquot of the urine was lyophilized in a tared vessel and the solid content determined. The remaining urine was reduced to about 50 ml. by evaporation in a low pressure still and then lyophilized. This permitted accurate calculation of the total dry residue and obviated the necessity for recovering all the dried urine in the larger specimen which clung tenaciously to the sides of the bottle in which it was lyophilized. Feces were dried over calcium chloride in a desiccator, ground to a fine powder in a Wiley mill, and mixed thoroughly.

* This study was aided by a grant from the American Society, on recommendation of the Committee on Growth of the National Research Council.

Received for publication May 18, 1951.

Some of the rats with tumors were allowed to die, but all other rats were killed by decapitation. The gastrointestinal tracts were removed, opened from gastric cardia to the anus, and cleaned. Each carcass was ground in an ordinary hand meat-grinder and then lyophilized. The dried tissue was ground in a Wiley mill until it would pass through a 20-mesh screen. Tumors were treated in the same way. The resulting powder was mixed according to procedures described by the American Society for Testing Materials (1). This method for producing a dry, powdered rat is satisfactory as long as the total lipid content of the mixture remains less than 30 per cent. Higher fat concentrations produce a pultaceous product which is difficult to mix.

Samples to be burned were made into pellets. At least 500 mg. of dried urine or feces were required for satisfactory analyses. Each pellet of dried rat or tumor weighed approximately 1 gm. All caloric measurements were made in a Parr adiabatic oxygen bomb calorimeter. Combustion values obtained from duplicate aliquots of the same specimen seldom varied by more than 2 per cent.

The conditions were the same in subsequent experiments with the following exceptions:

Most of the rats were housed in air-conditioned quarters maintained at 26 ± 1° C. and 50 per cent relative humidity. Each experimental group was larger, containing twelve to twenty rats, of which approximately five to seven were cancerous. Excreta were not collected.

The nitrogen content of all specimens was determined by titrimetric semi-micro Kjeldahl analysis with copper and selenium as catalysts. Total lipid of tissues and tumors was measured by a gravimetric method, with ethanol-diethyl ether and chloroform-methanol as solvents (2). The fat-free nitrogen concentration of rat carcasses, dissolved in an alcoholic solution of potassium hydroxide, was reported previously as 3.82 ± 0.06 per cent. No significant difference could be detected in the fat-free nitrogen concentration of normal rats and the carcasses of tumor-bearing rats (4). The new method of preparing tissues does not influence these results significantly, for the fat-free nitrogen content of 27 rats that died or were killed by nonexsanguinating methods was 3.74 ± 0.02 per cent. The corresponding value for 90 decapitated rats, however, which may lose as much as 10 gm. of blood, was 3.44 ± 0.01 per cent.

The caloric value of 1 gm. of rat varies with the sex and weight of the animal, being markedly influenced by dietary intake, since the caloric value of fat is so much higher than that of either carbohydrate or protein. The caloric value of 21 rats weighing from 156 to 241 gm. ranged from 1.77 to 2.58 calories per gram of wet weight. The mean value was 2.11 ± 0.5 calories per gram, a variation of almost 10 per cent. The caloric values of pair-fed rats, however, were more constant—e.g., 1.78 and 1.79, 2.08 and 2.14, 2.05 and 2.06, 2.16 and 2.18 calories per gram, respectively. Therefore, all calculations for each experiment were based on the caloric value of a normal rat ingesting identical amounts of the same diet eaten by the other two members of the group.

Noncancerous rats pair-fed to rats of the same age, weight, and sex that bear progressively growing Walker carcinoma 256 usually fail to gain and eventually lose weight if the food intake of the normal rats is based on milligrams of diet per gram of body weight ingested by its cancerous fellow (4). Further experience has shown that noncancerous rats that ingest identical amounts of the diet consumed by the tumor bearers may gain weight for a few days after inception of tumor growth. The calories represented by this increment need not be considered in calculating the calories lost in the course of tumor growth, for this study represents a determination of the over-all change. One source of error, however, is the variation in weight among the members of a group of pair-fed rats even though they are of the same age and sex. It has been assumed that rats are of comparable composition if the weight of the pair-fed rats does not diverge more than 5 per cent from that of the experimental animal during the course of normal growth. Observation of total nitrogen, total lipid, and total water content of such groups of pair-fed rats indicates that variation in these constituents is seldom greater than the variation in the total weights of the rats within each group. Nevertheless, values obtained from these experiments must be considered as relative rather than absolute.

RESULTS

The data obtained from each group of pair-fed rats are listed in Table 1.

There was little variation in the caloric value of the urine and feces within each group of pair-fed rats. The deviation between the control rat and the experimental rat varied from 1.1 to 6.4 per cent during normal growth (average 3.4 per cent). Variation during the course of tumor growth was from 0.5 to 14.9 per cent (average 5.3 per cent). Probably these variations are accounted for by technical difficulties, since there is no consistent tendency for the experimental rat to excrete either fewer or more calories than its pair-fed noncancerous control. This suggests that the nitro-

gen excretion of the two animals in each pair is not appreciably different, because nitrogenous constituents contribute the greatest part of the caloric value of urine. Supporting evidence is obtained from a comparison of the nitrogen content of each tumor-bearing rat and its pair-fed noncancerous control (Table 2). The differences observed are well within the limitations of the procedures used, except for group 1 in which the cancerous rat contained much more nitrogen than did its fellow. This is not an isolated instance, since a tendency for some tumor-bearing rats to store more nitrogen than do their pair-fed noncancerous controls has been observed in nitrogen balance experiments. It is more common, however, for the cancerous and noncancerous rats that eat identical amounts of the same diet to excrete approximately the same amount of nitrogen until late in the course of tumor growth.

The great variability in the cost of building a tumor is striking and appears to be caused solely by loss in total lipid. The caloric value of 16 samples of rat lipid extracted from different rat carcasses varied from 8.88 to 9.40 calories per gram with a mean of 9.11 ± 0.03 calories. By dividing the caloric loss from the carcass minus the caloric value of the tumor by the factor 9.11, a figure is derived for each tumor-bearing rat which represents the calculated lipid loss if all the caloric deficit corrected for the calories in the tumor resulted from combustion of fats. This figure agrees surprisingly well with the observed lipid loss obtained by subtracting the total lipid remaining in the tumor-bearing rat's carcass from the lipid present in the pair-fed control killed on the day that the first palpable tumor was noted. These results are listed in Table 3. There is more variation in similar data computed for the pair-fed noncancerous control rats.

No simple correlation between the rate at which the caloric deficits were acquired and the size of the tumors that grew was found. A larger deficit was attained by rat number 3a, which lived only 19 days, than was acquired by rat number 7a, which lived 32 days (Table 1).

TABLE 1

COMBUSTION VALUES AND WEIGHTS OF RATS BEARING WALKER CARCINOMA 256 AND THEIR NONCANCEROUS PAIR-FED CONTROLS

	Rat 1a tumor	Rat 1b control	Rat 2a tumor	Rat 2b control	Rat 3a tumor	Rat 3b control	Rat 4a tumor	Rat 4b control	Rat 5a tumor	Rat 5b control	Rat 6a tumor	Rat 6b control	Rat 7a tumor	Rat 7b control
Period of Normal Growth:														
Initial weight, gm.	157	151	160	164	122	129	173	185	158	169	153	154	165	156
Duration, days	21	21	17	17	14	14	14	14	21	21	35	35	14	14
Weight gain, gm.	65	69	40	41	66	60	58	51	35	32	87	88	49	51
Calories ingesta less calories excreta	1,016	972	673	694	627	634	827	880	922	945	1,634	1,696	821	802
Caloric value of third rat per gram, wet weight	2.14		2.24		2.47		1.99		2.27		2.03		2.15	
Period of Tumor Growth:														
Initial weight, gm.	222	220	200	205	188	189	218	224	193	201	240	242	214	207
Initial calories	475	471	448	459	464	467	434	446	438	456	487	491	460	445
Duration, days	12	12	14	14	19	19	21	21	23	23	23	23	32	32
Final carcass weight, gm.	204	212	173	200	125	182	158	205	134	191	175	248	154	209
Tumor weight, gm.	51		52		41		75		56		81		99	
Calories ingesta less calories excreta	410	412	547	517	646	586	654	658	565	556	757	870	1,008	1,041
Calories in rat carcass, final	315	454	270	476	179	425	234	394	161	419	222	487	186	384
Calories in tumor	38		43		35		64		50		63		85	
Net caloric change	−122	−17	−135	+17	−250	−42	−136	−52	−227	−37	−202	−4	−189	−61

It is reasonable to assume that the principal energy-producing foodstuffs, i.e., fat and carbohydrate, eaten by the rat during the period of tumor growth were burned some time during that period. Perhaps some superfluous carbohydrate and/or fat may have been stored for a time as depot fat, but the over-all loss of calories surely indicates that it must have been burned sometime between

TABLE 2

NITROGEN CONTENT OF TUMOR-BEARING RATS AND NONCANCEROUS PAIR-FED CONTROLS

TUMOR-BEARING RAT				PAIR-FED CONTROL	
Rat no.	Carcass nitrogen (gm.)	Tumor nitrogen (gm.)	Total nitrogen (gm.)	Rat no.	Total nitrogen (gm.)
1a	7.015	1.102	8.117	1b	7.030
2a	6.230	0.977	7.207	2b	7.230
3a	4.379	0.826	5.205	3b	5.667
4a	5.593	1.560	7.153	4b	7.120
5a	4.596	1.536	6.321	5b	6.338
6a	5.663	1.575	7.238	6b	7.760
7a	5.196	1.987	7.183	7b	6.951

the onset of progressive growth of the neoplasm and the death of the animal. One may disregard the influence of the potential calories derived from ingested protein for the purposes of this experiment, because the caloric values of ingesta minus excreta were not significantly different in each pair of rats. The amount of glycogen in a rat is so small that changes in its concentration probably could not be detected by our methods. The combustion values of the sugar and Crisco in the diet were found to be 3.88 and 9.30 calories per gram, respectively. The sum of the calories from carbohydrate and fat eaten during the period of tumor growth and the calories lost from the whole rat (rat plus tumor) during the same period represents an estimate of the calories derived from materials that serve primarily as sources of energy. These data are presented in Table 4. The over-all expenditure of calories from energy-producing substances, per gram of tumor formed, varied little for six of the seven rats. This suggests that a constant ratio may exist between growth of the Walker carcinoma and the energy requirements of the cancerous organism.

TABLE 3

COMPARISON OF CHANGE IN TOTAL LIPID CALCULATED FROM CALORIC DATA WITH OBSERVED CHANGE

Tumor-bearing rat			Pair-fed control		
Rat no.	Calculated (gm.)	Observed (gm.)	Rat no.	Calculated (gm.)	Observed (gm.)
1a	−13.4	− 8.1	1b	−1.9	− 1.5
2a	−14.8	−15.8	2b	+1.9	+ 0.2
3a	−27.4	−27.9	3b	−4.6	− 6.4
4a	−14.9	−14.6	4b	−5.7	− 6.0
5a	−25.0	−24.0	5b	−4.1	− 4.3
6a	−22.2	−21.6	6b	−0.4	− 2.4
7a	−20.8	−23.3	7b	−6.7	−11.3

This hypothesis was tested through estimating with bomb calorimetry the approximate energy expenditure of 21 additional rats, weighing from 200–225 gm. at the beginning of tumor growth, that died or were sacrificed when their tumors weighed between 4 and 103 gm. The combustion value of each rat plus its tumor was compared to that of a group of noncancerous pair-fed rats killed on the day that the first growth of the tumor was noted. Rats bearing relatively small tumors contained more calories than did pair-fed controls killed at the onset of tumor growth. Caloric increments were subtracted from, and caloric decrements added to, the combustion values of ingested fat and carbohydrate. The data indicated a regression of calories expended on tumor weight of the order: $y = a + bx$. Again, the caloric loss by the rat during the period of its tumor growth could be accounted for solely on the basis of loss of lipid under our experimental conditions.

Fifty-one additional male rats weighing from 180–200 gm. were killed when their tumors weighed from 4 to 115 gm. The difference between the lipid values of each animal at the time of death and its pair-fed noncancerous control killed at the inception of tumor growth was multiplied by the factor 9.11, the caloric value of rat lipid. This was taken as the caloric increment or deficit in the tumor-bearing rat and added algebraically to the caloric value of the ingested carbohydrate and fat. This group, treated independently, also indicated a constant relationship between caloric expenditure and tumor weight of the same order as that noted in the previous experiment, nor did the value for the regression coefficient differ significantly between the two groups.

Therefore, all 79 rats in this group of experiments were treated as one experiment for statistical purposes. The regression coefficient, as calculated by the sum of least squares method, was 8.57 ± 0.25 and the correlation coefficient 0.94.

It is important to define the period of tumor growth during which the excessive caloric expenditure occurs. Progressive weight loss from the carcass (rat minus tumor) has already been described in rats bearing Walker carcinoma 256 under our experimental conditions (4). Furthermore, this weight loss cannot be accounted for solely by reduction in total body lipids, since nitrogenous substances are translocated from body stores to cancerous tissue (4, 7). A group of nineteen male rats of the same age and weight were fed the same amount of diet eaten by another rat. The Walker tumor was transplanted into seven of the rats after seventeen of the group had gained weight at the same rate for a period of 4 weeks. Three noncancerous rats in the group were sacrificed on the day that the tumors appeared as palpable nodules and their lipid concentrations determined. The remaining animals were killed at suitable intervals during tumor growth, at least

TABLE 4

ENERGY DERIVED FROM CARBOHYDRATE AND FAT EXPENDED DURING TUMOR GROWTH

Rat no.	Calories eaten (Carbohydrate and fat only)	Net caloric loss	Tumor weight (gm.)	Cal. spent/tumor wt.
1a	470	122	51	11.6
2a	464	135	52	11.5
3a	387	250	41	15.5
4a	625	136	75	10.1
5a	471	227	56	12.5
6a	652	202	81	10.5
7a	988	189	99	11.9

one cancerous and one noncancerous rat being decapitated at the same time. The carcass weights and lipid content were related to those of the rats sacrificed at the onset of tumor growth. The results are presented in Chart 1. No significant change in carcass weight or lipid content is noted between the cancerous and noncancerous rats during the first 9 days of tumor growth. Thereafter, both gross carcass weight and lipid content decline steadily and progressively in the tumor-bearing animals. The weight and total lipid content of the noncancerous rats changes in the same direction as does the food intake. This experiment has been repeated several times with almost identical results, even though the different groups of rats ate quite differently. The onset of excessive loss of lipid occurs when the neoplasm has attained a critical mass, about 10 per cent that of the total body weight (rat plus tumor).

DISCUSSION

The loss of calories in cancerous rats is due to two factors: anorexia and tumor growth. Since the pair-fed noncancerous rat loses calories solely because of restriction in food intake, the caloric loss resulting from this factor is available. One may not use the figure with impunity, however, for the relatively normal rat is subject to but one stress, while the tumor-bearing rat is subject to at least two stresses—which, evidence suggests, are interdependent rather than otherwise.

Apparently, the entire caloric deficit acquired during growth of Walker carcinoma 256 can be accounted for by loss of body lipids. The weight lost from the carcass of the cancerous rat (rat minus tumor) is due to loss of protein and lipid (4). The protein depletion need not produce a heightened excretion of nitrogen, since a large proportion, if not all, the excessive protein loss represents a translocation of nitrogenous substances from normal to neoplastic tissue (4, 7). The lipids lost from the body during tumor growth must be burned completely, for no evidence of ketosis has been demonstrated. Ketonuria does not occur, nor does the titratable acidity of the urine increase as the neoplasm grows larger.

The excessive loss of lipid from the cancerous rat probably represents an increased energy requirement associated with growth of the Walker carcinoma, which should mean an increased metabolic rate. Determination of basal metabolic rate in individual rats imposes certain technical difficulties that we are not prepared to meet at the present time. The total caloric deficit acquired during tumor growth may be relatively large, but the rate at which it develops not only varies but may be relatively slow. The largest caloric deficit observed among the rats studied was 250 calories, which took 19 days to produce. If the excessive lipid loss mirrors the caloric loss, as these experiments indicate, one would expect to find a progressively increasing excess expenditure of energy as the tumor grows larger. The detection of small differences in oxygen consumption or heat elimination is difficult during short periods of observation and requires an apparatus of great precision.

The method used in estimating the energy expenditure of rats bearing Walker carcinoma 256 may be considered rather arbitrary, since it does not consider the contribution of protein to energy production. Confirmation of the suggested relationship between tumor mass and energy expenditure must be sought by some other technic. All attempts to introduce a temporal factor into the calculations have produced scatter diagrams, showing that those rats which eat the least accumulate their large caloric deficits rapidly. Conversely, the cancerous host that eats well loses calories slowly. Failure to establish a constant relationship among time, tumor growth, and energy expenditure may indicate that the growth rate of Walker carcinoma 256 is controlled by factors that are not essentially nutritional. Experience with groups of pair-fed rats of the same age, sex, and initial weight supports this view. The rats within each group ingest identical amounts of the same diet for at least 4 weeks prior to the onset of tumor growth, as well as throughout the cancerous period. They grow at the same rate before the neoplasms appear, as do most of the tumors that follow transplantation, but exceptions are encountered in almost every experiment. Such de-

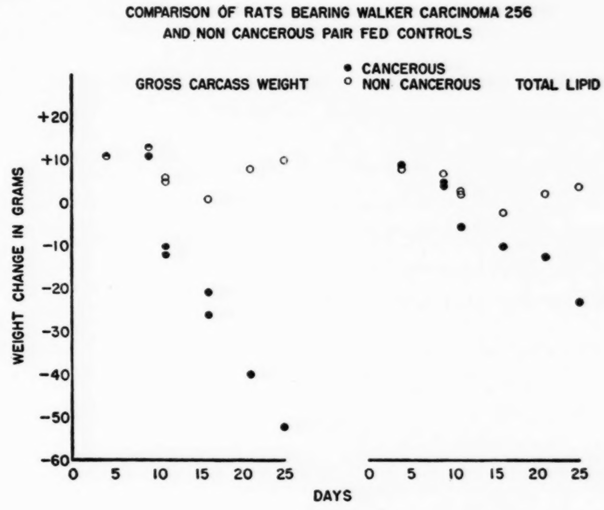


CHART 1

viations occur in both directions from the mean growth rate.

The caloric expenditure of tumor-bearing rats and their pair-fed controls may be compared on the basis of the estimated accumulated caloric output divided by the period of observation, expressed in days. Apparently in the noncancerous rats the caloric expenditure is decreased appreciably as food intake decreases. The metabolism of the cancerous animals apparently is reduced also, but at a slower rate. This suggests that progressive growth of the Walker carcinoma 256 prevents its host from adapting its economy to the stress of decreased dietary intake.

SUMMARY

Young, adult, male, albino rats in which Walker carcinoma 256 grew progressively lost more calories than did pair-fed noncancerous rats of the same age and initial weight. The caloric deficit measured by bomb calorimetry equalled the caloric value of the excess lipid lost by the cancerous animals. It was acquired only after the neoplasm had attained a critical mass and increased steadily thereafter.

The estimated expenditure by the host of energy derived from fat and carbohydrate during the period of tumor growth appeared to bear a constant relationship to the weight of the Walker carcinoma.

REFERENCES

1. A.S.T.M. Committee D-5, A.S.T.M. Standards on Coal and Coke. Philadelphia: American Society for Testing Materials, 1948.
2. Haven, F. L.; Bloor, W. R.; and Randall, C. The Fatty Acid Composition of Rats Growing Walker Carcinoma 256. Cancer Research, **11**: 619–23, 1951.
3. Mider, G. B.; Sherman, C. D., Jr.; and Morton, J. J. The Effect of Walker Carcinoma 246 on the Total Lipid Content of Rats. Cancer Research, **9**:222–24, 1949.
4. Mider, G. B.; Tesluk, H.; and Morton, J. J. Effects of Walker Carcinoma 256 on Food Intake, Body Weight and Nitrogen Metabolism of Growing Rats. Acta de l'Union internationale contre le Cancer, **6**:409–20, 1948.
5. Rubner, M. Calorimetrische Untersuchungen. Ztschr. f. Biol., **21**:250–334, 337–410, 1885.
6. ———. Die Quelle der thierischen Wärme. *Ibid.*, **30**: 73–142, 1894.
7. Sherman, C. D., Jr.; Morton, J. J.; and Mider, G. B. Potential Sources of Tumor Nitrogen. Cancer Research, **10**:374–78, 1950.

The Virus-induced Papilloma-to-Carcinoma Sequence

IV. Carcinomas in Domestic Rabbits Infected while *in Utero**

ROBERT G. FISCHER† AND JEROME T. SYVERTON‡

(*†Department of Bacteriology, University of North Dakota, Grand Forks, N.D., and ‡Department of Bacteriology, University of Minnesota, Minneapolis, Minn.*)

The growth pattern of the epithelial tumor induced by the rabbit papilloma virus (Shope) (11) is characterized by a sequence of changes that is well defined and predictable (4). Three successive growth phases occur: proliferative, stationary, and involutionary. The involutionary phase can terminate either in regression and disappearance, or in cancer. Since earlier published studies of the virus-induced papilloma and of the cancer that may follow (1-11, 13, 15) left open the comparative incidence of malignancy in the two host species, it was of particular interest to learn (15, 16) that cancer occurred without interference with the natural sequence of alterative changes in about 25 per cent (32 out of 137) of the cottontail rabbits and in about 75 per cent (22 out of 30) of the domestic rabbits that had been under observation for 6 months or longer. Moreover, it became apparent from the results of this same study (15), and from the other two communications in this series (16, 17), that papilloma virus can operate as a remarkably efficacious carcinogenic agent, for both domestic and cottontail rabbits. This observation made it desirable to select for experimental study certain factors that may operate to condition the papilloma-to-carcinoma sequence or to influence differences in species and host reactivity. Among these factors is the age of the host. Since age is recognized commonly as such an exceedingly important determinant for conditioning the extent and type of response to infectious, immunogenic, and carcinogenic agents, the present experimental study was carried out to learn what influence the immaturity of the host might exert upon the papilloma-to-carcinoma sequence. For this purpose fetal rabbits, while still *in utero*, were inoculated intracutaneously at multiple sites with papilloma virus. These fetal rabbits were kept under observation after birth so as to record for each animal (*a*) the time of appearance of papillomatous lesions, (*b*) any differences from the sequence of changes which had been observed for 167 mature rabbits (15), and, particularly, (*c*) the time interval for the development of cancer. The findings of this study are contained in the present paper.

* This investigation was aided by grants from the American Cancer Society, on recommendation of the Committee on Growth, National Research Council, and the North Dakota Cancer Society, Inc., Division of the American Cancer Society.

Received for publication May 28, 1951.

MATERIALS AND METHODS

Virus.—The papilloma virus (Shope) was obtained from papillomas that had been incurred under natural conditions in Kansas by cottontail rabbits (genus Sylvilagus). The papillomatous tissue was kept in 50 per cent neutral glycerol at 4° C. until it was made ready for transfer either to fetal or to control adult rabbits. The tissue was throughly triturated with alundum in 0.85 per cent NaCl solution to yield a 10 per cent suspension. This suspension was centrifuged in an angle-head at 2,000 r.p.m. for 30 minutes and the supernatant fluid withdrawn for inoculation.

Rabbits.—Fetal rabbits, provided by purebred American Dutch does, were employed as the test animal. Since the period of gestation for domestic rabbits (genus Oryctolagus) is 30 days, the does were bred and subjected 20 days thereafter to a laparotomy when their fetuses were from 18 to 20 days of age.

Method of inoculation.—The mother rabbit was placed on its back with its legs extended and fastened by ties to an operating table; the abdominal region was shaved, cleansed with soap and water, and zephiran chloride was applied. Deep anesthesia was induced and maintained by utilizing in combination sodium pentothal intravenously and ether by inhalation. The bicornuate uterus

with its contained fetuses was exposed quickly by making a 2-inch left rectus incision extending from the level of the umbilicus to the symphysis pubis. Since it was impractical to employ the cross-hatch scarification technic for transfer of papilloma virus for the inoculation of embryos *in utero*, another method was devised. The approach consisted of grasping between the thumb and forefinger each uterine horn with its contained fetuses, so as to make known the bodily configuration of each fetus. A 26-gauge needle attached to a 1-ml. syringe containing 0.5 ml. of virus suspension made it possible to pierce the uterine wall and to make multiple insertions into the underlying fetal skin. It can be assumed that the virus was thereby brought regularly into contact with injured epithelial cells. Each embryo was inoculated in this manner at multiple sites for a total of approximately 0.5 ml. of virus suspension.

The female rabbits usually gave birth to litters within 10 days of injection. However, some does aborted, and other rabbits practiced cannibalism to destroy their entire litter soon after birth.

RESULTS

The offspring that were inoculated while *in utero* were kept for observation of the papilloma-to-carcinoma sequence. The successive changes were found to conform to the growth pattern in domesticated rabbits (15).

None of the young at birth exhibited any signs of skin injury or papillomatous growth. However, in from 7 to 10 days after birth or from 17 to 24 days after inoculation, discrete papillar growths appeared. Many of these papillomas merged to form aggregative masses. None of the animals was devoid of growths. The final number of papillomatous lesions on single animals ranged from three to seventeen. These papillomas proliferated for 3 months. Lateral extension subcutaneously of the papillomatous growths was observed in two of the animals. The proliferative phase was followed by the stationary, or quiescent, stage. Alterative changes began to appear about the fifth month to result during the succeeding 3 months in carcinomatous degeneration in lesions of five of the hosts. It is of interest that none of the papillomas was observed to regress completely, although a number showed marked diminution in size. The six rabbits that survived for 6 months or longer provided tissues at biopsy for histopathological study. Five of the six rabbits within 7 months after injection yielded tumors that were proved microscopically to be epidermoid carcinomas, and the sixth rabbit was observed to have lesions undergoing degenerative changes during its eighth month of infection. However, these lesions were not established microscopically as cancerous when the rabbit died from intercurrent injection. The rabbits are described below in the order of their survival.

Rabbit no. 1.—Rabbit no. 1 presented on examination 14 days following its birth three papillomatous growths which measured 0.3 cm. in diameter and 0.3 cm. in height. By the sixth week, eight papillomas, ranging in size from $1 \times 1 \times 1$ cm. to $1.8 \times 2 \times 2$ cm., were noted. Three of these papillomas coalesced during the next 30 days to form a large papillomatous mass measuring $7 \times 5 \times 5$ cm. It was during the fifth month that this large growth underwent a change in appearance: necrosis occurred, the base of the growth appeared fleshy, and extension into the subcutaneous tissues was noted. Similar changes were observed in another papilloma. These two growths, when removed at autopsy and when subjected to microscopic examination, were proved to be epidermoid carcinomas (Figs. 1–5).

Rabbit no. 2.—Evidence of early papillomatous growths was noted on Rabbit no. 2, 15 days after birth, when each of three lesions measured approximately $0.2 \times 0.2 \times 0.2$ cm. On examination 2 weeks later, six papillomas were present, ranging in size from 0.2 to 1 cm. in diameter and from 0.2 to 2 cm. in height. Five weeks following birth, eight papillomatous growths were recorded. These papillomas grew progressively to range in size by the fifth month from $3 \times 3 \times 4$ cm. to $4.5 \times 4.5 \times 5$ cm. During the succeeding 60 days, the tumors underwent necrosis and for one an extension peripherally was noted. One of the tumorous growths was observed, during the seventh month, to have undergone a radical transformation in appearance. This lesion was sectioned at biopsy and found on microscopic examination to be an epidermoid carcinoma (Figs. 6–9).

Rabbit no. 3.—Three newly appeared papillomas were present on examination of Rabbit no. 3, 2 weeks after birth. No additional papillomas were noted during the 7 months that this rabbit was under observation. By the end of the sixth month, the papillomas had increased in size to $5 \times 3 \times 2.5$ cm., and one of the three growths had undergone necrosis, revealing a fleshy base and evidence of extension peripherally into the subcutaneous tissues. This tumor was examined at biopsy when the rabbit was 7 months old to yield evidence on microscopic examination of malignancy.

Rabbit no. 4.—Three papillomas were noted on examination 14 days after birth, and four were recorded when the rabbit was 5 weeks of age.

These papillomas increased in size to reach 3 × 2.5 × 3.5 cm. It was during the sixth month that one of the tumors on the back of the rabbit began to show degenerative changes. The surface growth sloughed, the base became fleshy, and evidence was noted of extension into the underlying tissues. The central part of the papillomatous tumor underwent necrosis. The degenerative lesions were removed at biopsy during the seventh month and shown by microscopic examination to be carcinomatous.

Rabbit no. 5.—Rabbit no. 5 had four papillomas 21 days following birth. During the next 60 days these four papillomas grew to reach a size approximating 2.5 × 2 × 4 cm. It was during the fifth and sixth months that evidence of extension and necrosis was recorded. A single lesion underwent further changes. It was removed at biopsy 30 days later and examined microscopically. A histologic diagnosis of epidermoid carcinoma was made.

Rabbit no. 6.—The papillomas on Rabbit no. 6 were limited to two growths that were observed first 37 days after birth of the rabbit. These tumors measured in diameter 0.4 cm. and 0.1 cm., respectively. Four months later the greatest size was reached when both tumors were 2.5 × 3.0 × 2.5 cm. No change in size or appearance occurred in the next 60 days. However, the tumors became dry during the seventh and eighth months and underwent a reduction in size to 1.5 × 1.0 × 1.0 cm. and 2.2 × 1.3 × 1.0 cm., respectively. The rabbit died from pasteurellosis in the eighth month following infection. The lesions on microscopic examination were benign hyperplastic, keratinized epithelial growths without evidence of malignant degeneration.

DISCUSSION

The observations recorded in the present paper make known the sequence of changes and the ultimate fate for experimentally induced papillomas that were induced by the transuterine injection of papilloma virus into the skin and other tissues of fetal rabbits (genus Oryctolagus). Epidermoid carcinomas resulted at one or more of the sites which had been occupied by virus-induced papillomas in five of the six rabbits that survived for from 5 to 7 months. The sixth rabbit in the group died of intercurrent bacterial infection 8 months after inoculation. The two papillomatous lesions on this rabbit had arisen after a long incubation period and had grown slowly to reach the regressive phase when examined microscopically.

The findings summarized above make it apparent that the susceptibility of domestic laboratory rabbits is not unduly influenced by the immaturity of the host. Thus, the results in this series of animals show that the immature rabbit is fully receptive to infection with papilloma virus and, particularly, to the development of the successive changes that may result ultimately in an epidermoid carcinoma. The evidence in support of this conclusion is the observation that five of the six animals in this study harbored lesions that underwent carcinomatous degeneration, whereas in similar studies (15) employing fully mature adult rabbits of a similar purebred line only eight of fourteen which had been kept under observation for from 6 to 12 months provided lesions that had undergone malignancy.

This study did not produce evidence for any significant alteration in the host cell-papilloma virus relationship. Thus, the injection of large amounts of papilloma virus into skin of fetal rabbits did not result, as anticipated, in widespread dissemination, or in evidence for the infection of cells other than of skin. The possible bearing upon these findings of earlier studies by Rous and Beard (1, 8, 9) is worthy of comment, since the results of these investigators may be interpreted (9) as evidence that embryonic epithelium is less susceptible and responsive than adult skin. However, the failure of papilloma virus in embryonic tissues which had been kept in contact with papilloma virus *in vitro* for from 30 to 120 minutes and implanted subcutaneously to show evidence for proliferation is not comparable to studies carried out *in vivo*. Other evidence (9) to suggest for immature epithelial cells a limited receptivity and response was the observation that the inoculation of domestic rabbits within a few days after birth produced less vigorous growth than resulted from the same inoculum in adult animals. However, this difference in age reactivity was not apparent when adult and newborn rabbits were tested for reactivity by employing for injection by cross-hatch scarification serial fivefold dilutions of a single inoculum.[1] Finally, the observation by Smith, Kidd, and Rous (12) that the presence of papilloma virus was made known by the transplantation of a squamous-cell carcinoma to newborn rabbits, but not on test in adult rabbits, can be interpreted as circumstantial evidence of differences between adult and newborn rabbits.

It is of interest that the early age at which infection occurred was without apparent influence on the unexplained phenomenon known as the "masking" effect. This phenomenon is related to host species, since virus from domestic rabbit papillomas is rarely recoverable in contrast to the

[1] J. T. Syverton, unpublished observations.

natural host, the cottontail rabbit, from which virus is readily recoverable.

SUMMARY

Neoplasms developed in rabbits which were infected *in utero* as readily as in mature animals. Thus, fetal domestic rabbits (genus Oryctolagus) were found fully susceptible to infection by papilloma virus, as manifested after birth by the papilloma-to-carcinoma sequence. The immaturity of the host was without any remarkable alterative effect in this sequence. Since the observation that cancer developed in five of six animals within 7 months of birth makes it apparent that the time required in immature rabbits for the papilloma-to-carcinoma sequence to eventuate in cancer is probably shorter than in adult rabbits, it is more than suggestive that the provocative carcinogenic effect of papilloma virus results from the initial host cell-virus relationship and that it is not significantly conditioned by the age of the host.

REFERENCES

1. Beard, J. W., and Rous, P. Virus-Induced Mammalian Growth with the Characters of a Tumor (Shope Rabbit Papilloma). II. Experimental Alterations of Growth on Skin: Morphological Considerations: The Phenomena of Retrogression. J. Exper. Med., **60**:723–40, 1934.
2. Höra, J. Shope-Papillom und Shope-Carcinom (spontane und experimentelle Unwandlung der Papillome in Krebs). Ztschr. f. Krebsforsch., **47**:303–24, 1938.
3. Kidd, J. G. Course of Virus-Induced Rabbit Papillomas as Determined by Virus, Cells, and Host. J. Exper. Med., **67**:551–74, 1938.
4. Kidd, J. G., and Rous, P. Cancers Deriving from Virus Papillomas of Wild Rabbits under Natural Conditions. J. Exper. Med., **71**:469–94, 1940.
5. Ladewig, P. Über das Shopesche Cottontail-Rabbit-Papilloma. Schweiz. Med. Wchnschr., **67**:165–68, 1937.
6. Oberling, C. The Riddle of Cancer (translated by W. H. Woglom), p. 196. New Haven: Yale University Press, 1944.
7. Peyron, A., and Poumeau-Delille, G. L'histopathologie et les modalités évolutives de la tumeur cutanée de Shope chez le lapin. Bull. Assoc. franç. p. l'étude du cancer, **28**:180–94, 1939.
8. Rous, P., and Beard, J. W. Virus-Induced Mammalian Growth with the Characters of a Tumor (The Shope Rabbit Papilloma). I. The Growth on Implantation within Favorable Hosts. J. Exper. Med., **60**:701–22, 1934.
9. ———. Virus-Induced Mammalian Growth with the Characters of a Tumor (The Shope Rabbit Papilloma). III. Further Characters of the Growth: General Discussion. J. Exper. Med., **60**:741–66, 1934.
10. ———. Carcinomatous Changes in Virus-Induced Papillomas of Skin of Rabbit. Proc. Soc. Exper. Biol. & Med., **32**:578–80, 1935.
11. Shope, R. E., and Hurst, E. W. Infectious Papillomatosis of Rabbits, with Note on the Histopathology. J. Exper. Med., **58**:607–24, 1933.
12. Smith, W. E.; Kidd, J. G.; and Rous, P. The Disappearance of the Rabbit Papilloma Virus (Shope) from the Carcinomas That Originate from Papilloma Cells. Fourth Internat. Cancer Research Congress, Abstracts of Communications, Vol. **84**, 1947.
13. Syverton, J. T. The Papilloma-to-Carcinoma Sequence in the Cottontail (Sylvilagus) Rabbit. Third Internat. Congress for Microbiology, Abstracts of Communications, New York, Section III, 341–42, 1939.
14. Syverton, J. T., and Berry, G. P. Carcinoma in Cottontail Rabbit Following Spontaneous Virus Papilloma (Shope). Proc. Soc. Exper. Biol. & Med., **33**:399–400, 1935.
15. Syverton, J. T.; Dascomb, H. E.; Koomen, J. Wells, E. B.; and Berry, G. P. The Virus-induced Papilloma-to-Carcinoma Sequence. I. The Growth Pattern in Natural and Experimental Infections. Cancer Research, **10**:379–84, 1950.
16. Syverton, J. T.; Dascomb, H. E.; Wells, E. B.; Koomen, J.; and Berry, G. P. The Virus-induced Rabbit Papilloma-to-Carcinoma Sequence. II. Carcinomas in the Natural Host, the Cottontail Rabbit. Cancer Research, **10**:440–44, 1950.
17. Syverton, J. T.; Wells, E. B.; Koomen, J.; Dascomb, H. E.; and Berry, G. P. The Virus-induced Rabbit Papilloma-to-Carcinoma Sequence. III. Immunological Tests for Papilloma Virus in Cottontail Carcinomas. Cancer Research, **10**:474–82, 1950.

The microscopic sections were stained with hematoxylin and eosin. The photographs were made by Mr. Henry Morris.

Fig. 1.—Tumor 1 of Rabbit 1, to show juncture where cancer arises from adjoining gnawed-off base of a benign papilloma. The underlying tissues are infiltrated and replaced by cancerous tissue. ×10.

Fig. 2.—The carcinoma of Figure 1 a few millimeters below the surface photographed to show total replacement by cancerous tissue. ×125.

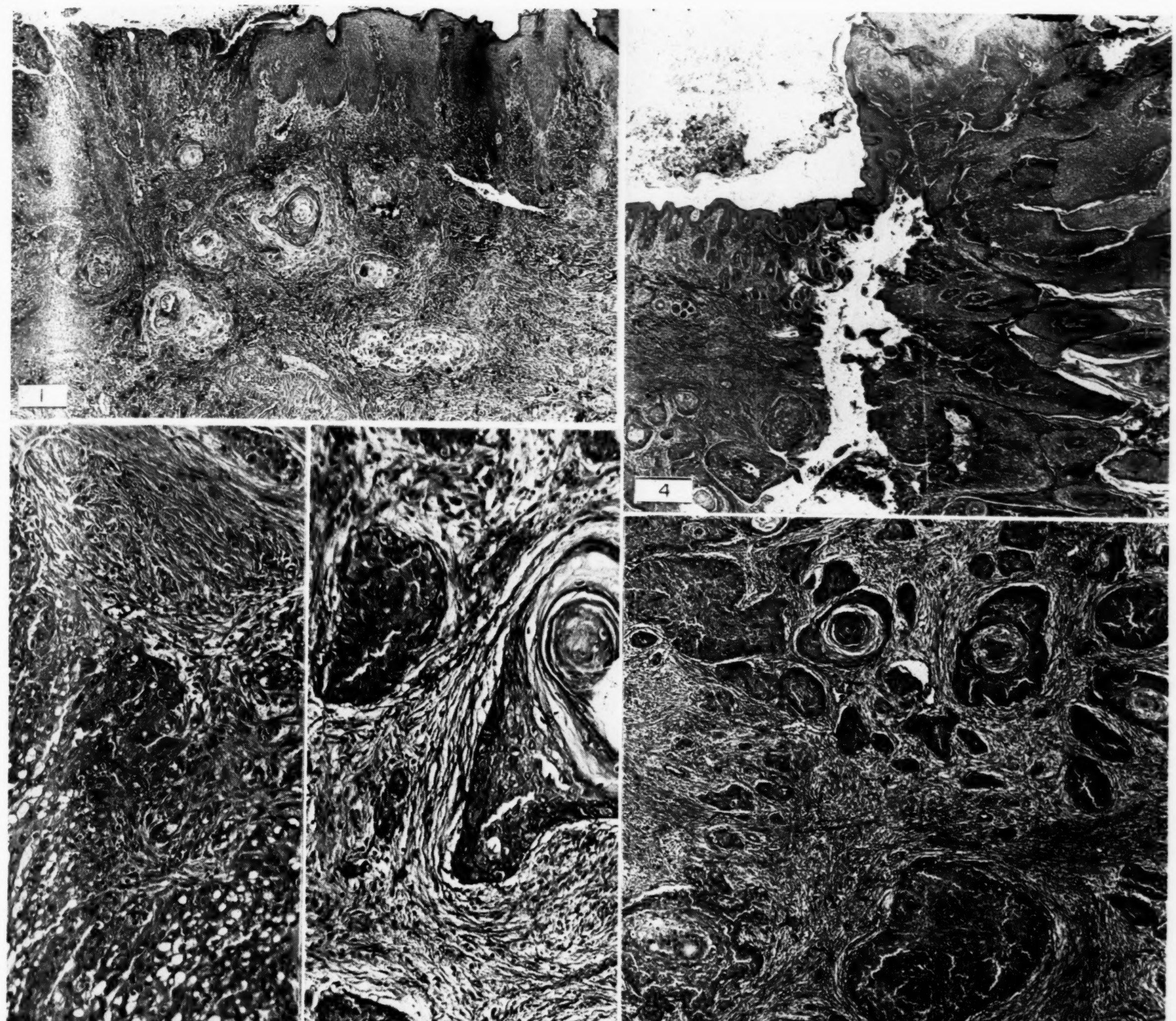
Fig. 3.—Another portion of the cancer of Figure 1. A squamous-cell carcinoma is seen. ×125.

Fig. 4.—Tumor 2 of Rabbit 1, to show the gnawed base of a virus papilloma (upper left), a squamous-cell carcinoma (lower left), and beyond the artifact a malignant papilloma (right half). ×10.

Fig. 5.—A section showing the tissues underlying the malignant papilloma of Figure 4. An epidermoid carcinoma is seen. ×32.

Fig. 6.—Tumor 1 of Rabbit 2. The overlying virus papilloma shows evidence of regression. The underlying tissues are largely replaced by a squamous-cell carcinoma. ×10.

Figs. 7, 8, and 9.—Figure 7 (×32) shows better the squamous-cell carcinoma immediately underlying the residual of the virus papilloma; Figure 8 (×125) shows at a higher magnification the adjoining cancer to the right; Figure 9 (×32) the cancer to the left.


1
2
3
4
5
6
7
8
9